ISSN 1346-9894

水研センター研報
Bull.Fish.Res.Agen.

水産総合研究センター
研究報告

40
平成27年3月

BULLETIN OF FISHERIES RESEARCH AGENCY

No. 40
March 2015

NOAA IRC Library
Pacific Islands Fisheries Science Center
Received 30 Jun 2015

独立行政法人 水産総合研究センター
神奈川県横浜市
FISHERIES RESEARCH AGENCY
Yokohama, Kanagawa, Japan

Digitized by the Internet Archive
in 2026

https://archive.org/details/bulletin-japan-fisheries-research-education-agency_2015-03_40

水産総合研究センター研究報告

第40号　　平成27年３月

目　次

(シンポジウム)

Pacific Bluefin and North Pacific Albacore Tuna Age Determination Workshop …… 1

The 41st Scientific Symposium of the UJNR Aquaculture Panel
"Advanced Aquaculture Technologies" …… 19

水研センター研報，第40号，1－11，平成27年
Bull. Fish. Res. Agen. No. 40, 1－11, 2015

A manual for age determination of Pacific bluefin tuna *Thunnus orientalis*

Tamaki Shimose[*1] and Taiki Ishihara[*2]

Abstract: Age determination techniques for Pacific bluefin tuna *Thunnus orientalis* using sectioned otoliths have been developed in recent years and a protocol of age determination is described. Opaque zones close to the core in the sectioned otoliths are difficult to recognize and some criteria are provided. The distance from the core is useful to recognize the 1st and 2nd opaque zones. The 1st to ca. 9th opaque zones are formed with similar distances between opaque zones. After ca. 9th opaque zones are relatively easy to recognize. This information facilitates age and growth studies of Pacific bluefin tuna which is used for stock assessment of this valuable fish species.

Key words: age determination, Pacific bluefin tuna, sectioned otolith, *Thunnus orientalis*

Pacific bluefin tuna *Thunnus orientalis* is one of the most highly valued fishery resources and is caught over a wide area in the North Pacific Ocean. Stock assessment of the species is conducted every two years using age-related information such as growth parameters, age composition of the catch by fishing ground, and fishing mortality etc. Age and growth of the species have been estimated by vertebral ring counts (Aikawa and Kato, 1938), scale ring counts, modal progression analysis (Yukinawa and Yabuta, 1967), tag release-recapture analysis (Bayliff *et al.*, 1991) and otolith micro-increment analysis (Foreman, 1996). These age and growth studies have obtained maximum ages up to 10 years old for fish up to 200 cm fork length (FL). After the development of age determination technique using sectioned otoliths for southern bluefin tuna *Thunnus maccoyii* (Kalish *et al.*, 1996; Clear *et al.*, 2000) and Atlantic bluefin tuna *Thunnus thynnus* (Neilson and Campana, 2008), the sectioned otolith method is recognized as the most reliable tool to age these tuna species (e.g. Gunn *et al.*, 2008).

Initial age determination of Pacific bluefin tuna using sectioned otoliths referred to the manual for southern bluefin tuna (Anonymous, 2002). However, age-length plots of Pacific bluefin tuna were not smooth when criteria for identification of otolith opaque zones of southern bluefin tuna was used (Tanabe and Kai, 2007). Specific criteria for Pacific bluefin tuna otoliths was subsequently developed (Shimose *et al.*, 2008, 2009), and age estimates from 1 to 26 years old were obtained. To share the age determination technique for Pacific bluefin tuna, the International Scientific Committee recommended the development of a technical manual, focusing on the interpretation of annual bands in the sectioned otolith. The international workshop "Pacific Bluefin and North Pacific Albacore Tuna Age Determination Workshop" was held during 13–16 November 2013, and age determination protocol for Pacific bluefin tuna was discussed. In this manual, current methods of sample collection, otolith preparation, criteria to identify annual opaque zones, and other technical notes are described as an output of the workshop.

Collecting individual data and otolith samples

In 1992, the National Research Institute of Far Seas Fisheries started collecting otolith samples from Pacific bluefin tuna. The collecting effort

2014年6月13日受理（Received on June 13, 2014）
*1 Research Center for Subtropical Fisheries, Seikai National Fisheries Research Institute, Fisheries Research Agency, 148-446, Fukai-Ohta, Ishigaki, Okinawa, 907-0451 Japan.
*2 National Research Institute of Far Seas Fisheries, 5-7-1, Orido, Shimizu, Shizuoka,424-8633, Japan

greatly increased from 2007 and approximately 200 to 1,000 (depending on the year) otolith samples are currently collected each year. The sampling protocol was divided into three methods (Fig. 1).

1) Whole fish is purchased at the fishing port, and taken to the laboratory where the length is measured and otoliths removed. The cost of purchasing the fish is relatively high, so this

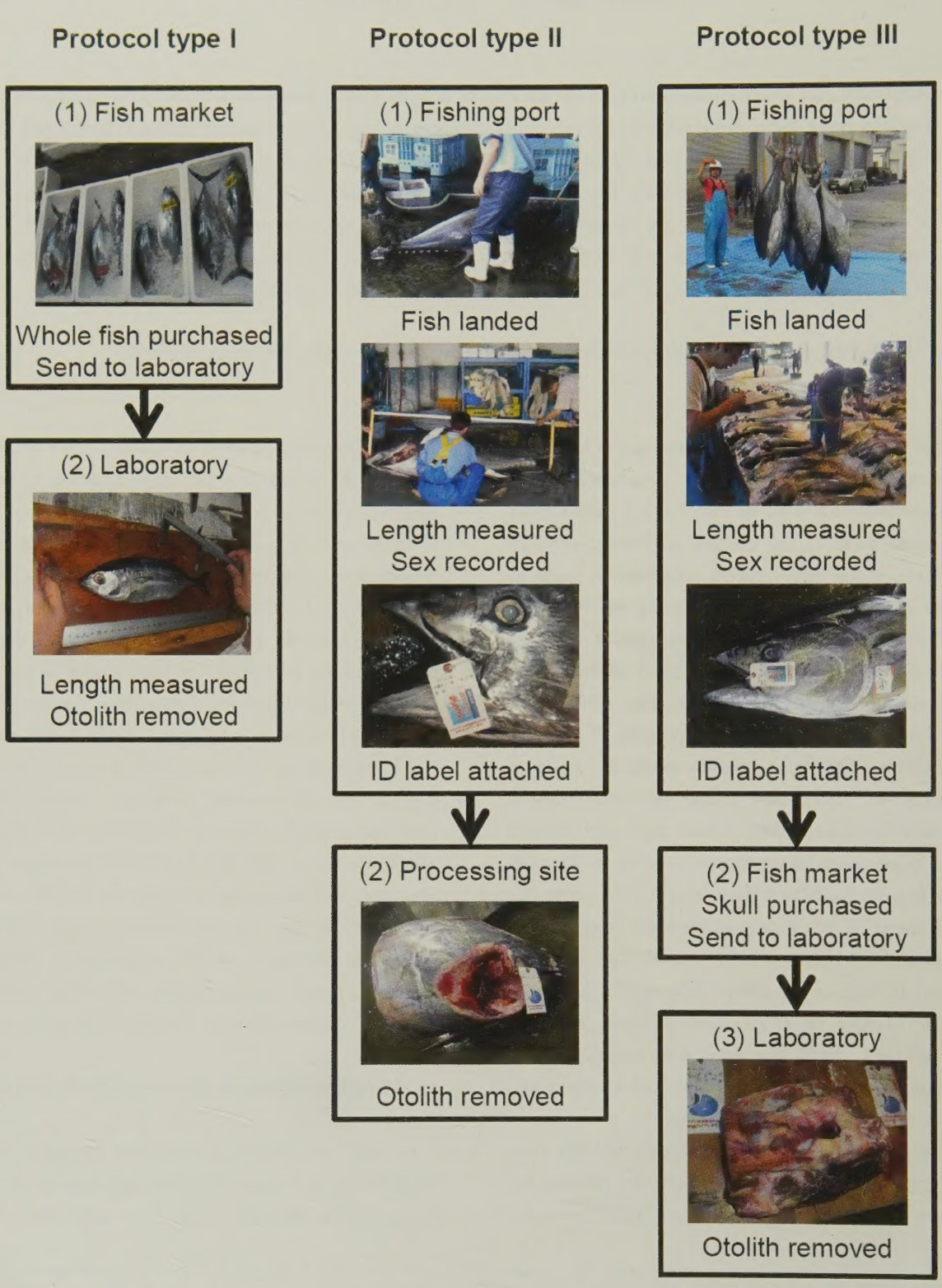


Fig. 1. Three types of sampling methods to collect length data and otoliths of Pacific bluefin tuna *Thunnus orientalis*.

method is generally only applicable to small individuals (ca. <100 cm FL).

2) Landed fish is measured, sexed and labeled at the fishing port, and then taken to the public fish processing site where the otolith is collected from the labeled skull a few days later.
3) Landed fish is measured, sexed and labeled at the fishing port, and then sent to Tsukiji fish market. The labeled skull is purchased from the fish buyer or processor at the market. This method is costly but less so than for the first method for large individuals.

To remove the otoliths, the skull of Pacific bluefin tuna can be cut using a large saw at the top of head using either a flat or V-shaped cut. The brain tissues are removed and the otoliths are extracted with the otolith membrane still attached. Otoliths should be cleaned using distilled water before storing dry in vials. Note that otoliths should not be cleaned/stored in tap water (which includes chlorine), seawater, or formalin because they may damage the otolith. There are three pairs of otoliths found in the skull but only the largest "sagittal pair" is used for age determination (Fig. 2).

Sampling strategies relating fish size

Two sampling strategies are suggested for southern bluefin tuna; i.e. length stratified sampling and random sampling (Anonymous, 2002). Length stratified sampling is preferable to derive growth curves or develop age-length keys for all length classes, while random sampling is useful to estimate the age composition of the catch of a specific fishery or area (Anonymous, 2002). Small Pacific bluefin tuna (<100 cm) can be selected by length and purchased as whole fish. Middle- (100–200 cm) or large- (>200 cm) sized fish can be sampled independently, because the landing ports of these two size classes are slightly different from each other. However, only random sampling is possible within each of these two size classes because specific buyers send the skulls and which skulls will be taken cannot be predicted. Selecting otoliths from those collected for age estimation can be undertaken using a stratified approach.

There are many fishing grounds and fishing gear types for Pacific bluefin tuna in Japan. Some examples of major fishing areas and fisheries are follows (Fig. 3):

A) Tsugaru Strait: troll and longline vessels target

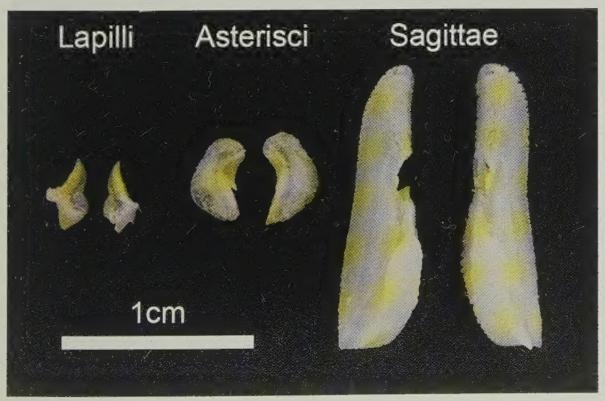


Fig. 2. Photograph of three pairs of otoliths of Pacific bluefin tuna *Thunnus orientalis* (141 cm FL, male). The largest "sagitta" is used for age determination.

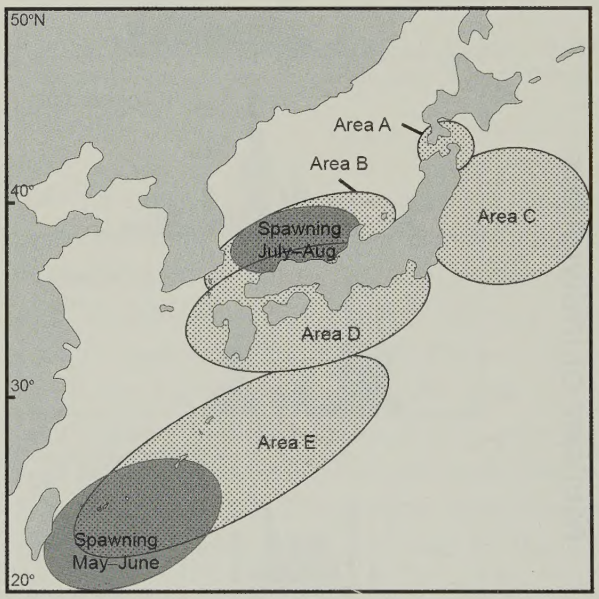


Fig. 3. Map of spawning grounds and fishing grounds of Pacific bluefin tuna *Thunnus orientalis*. Two spawning grounds are known and peak spawning seasons are shown. Major fishing grounds in Japan are divided into five areas; i.e. A) Tsugaru Strait: operating troll and longline fisheries during August to December, B) Sea of Japan: operating purse seine fishery during June to August, C) Off Sanriku: operating purse seine and set net fisheries year round, D) Southern Japan: operating troll fishery during July to December, E) Nansei Islands: operating longline fishery during April to July.

middle- to large-size fish during August to December, with a small number of landings in the other months.

B) Sea of Japan: purse seine vessels target small to middle-size fish during June to August, with minor landings in the other months. Most of middle-size fish are in spawning condition (Okochi *et al.*, unpublished data).

C) Off Sanriku: purse seine and set net vessels occasionally catch small to middle-size fish.

D) Southern Japan: troll vessels target small-size fish in July to December, with minor landings in the other months.

E) Nansei Islands: longline vessels target large-size fish in April to July. These fish are in spawning condition (Okochi *et al.*, unpublished data).

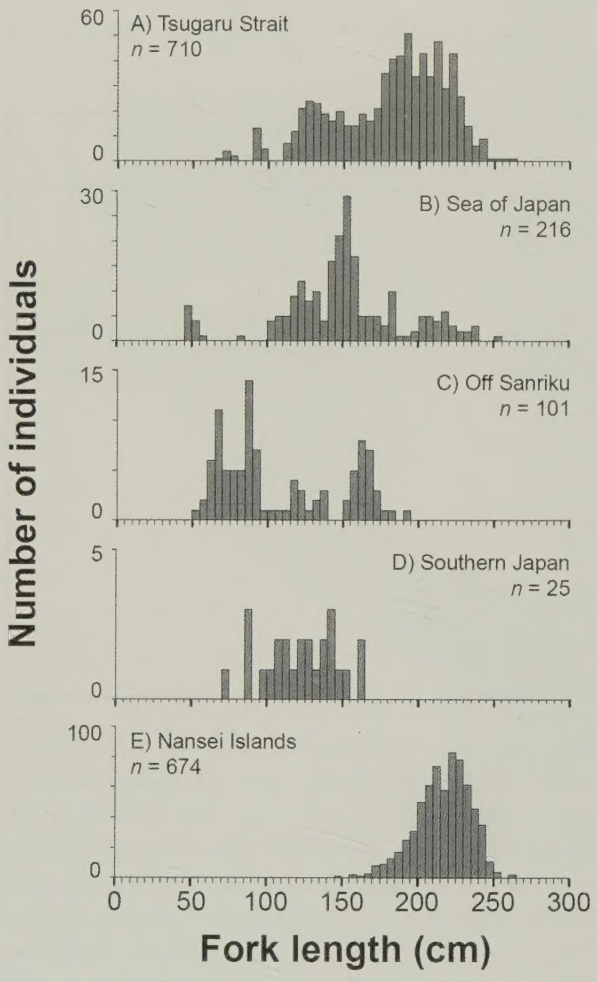


Fig. 4. Fork length frequency distribution of Pacific bluefin tuna *Thunnus orientalis* at five different fishing grounds. Data are from otolith samples collected in Japan between 1992 and 2011.

Although fork length frequency distributions differ among areas, the combined data and samples cover the full size range of fish to derive growth curves (Fig. 4). Although there are many other fisheries not only in Japan but also in other countries, details are not shown here.

Preparation of otolith section

Structures and terminology for tuna otoliths are well described in the ageing manual for southern bluefin tuna (Anonymous, 2002), and also shown here using Pacific bluefin tuna otoliths (Fig. 5). The rostrum is situated on the ventral side of the otolith and extends anteriorly. The core is difficult to recognize on the otolith surface, but is located just anterior to distal lobe. The sulcus is situated on the inner side of the fish body between the dorsal arm and ventral arm on the otolith.

Each otolith is embedded in epoxy resin and allowed to harden for 24 hours before sectioning. Then it is sectioned ca. 0.3–0.5 mm thick in an area including otolith core region (Fig. 5). Sectioning should be conducted perpendicular to the longest

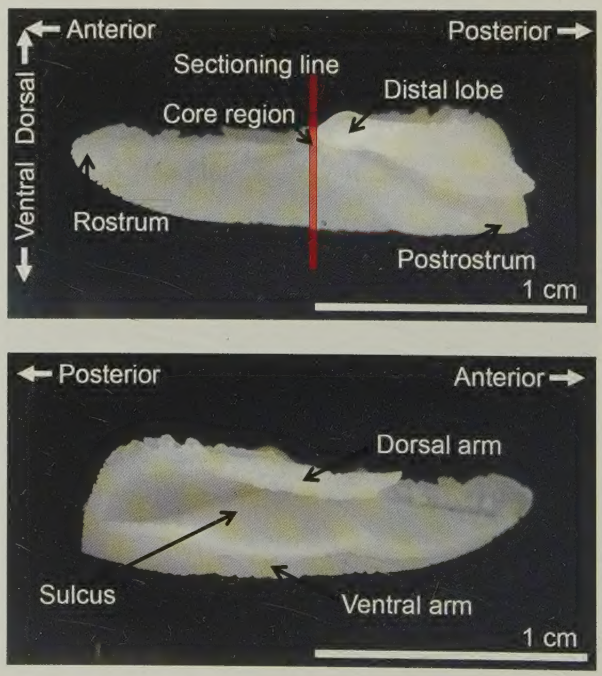


Fig. 5. Photographs of distal side (upper) and sulcus side (lower) for whole left otolith of Pacific bluefin tuna *Thunnus orientalis* (243 cm FL male).

axis of whole otolith as precise as possible. Preparation of serial sections around core region is recommended to refer to if there is low readability in a primary section. The thinner sections show the clearest view but they can be easily broken. Some sectioning methods are described in the manual for southern bluefin tuna (Anonymous, 2002). The section is attached on a glass slide using mounting medium, such as Crystal Bond (melts at low temperature heat) or Marinol (dissolves in solvent). The surface of the attached otolith should be polished flat using aluminum powder or polishing paper, or coated with a liquid mounting medium. The remaining otolith pieces encased in resin block should be kept after sectioning for possible use later.

Observation of sectioned otolith

Prepared slides of otoliths are viewed under a stereo microscope using reflected light and a black background or using a compound microscope equipped with transmitted light. Using reflected light is beneficial for checking whether the otolith edge condition is opaque or translucent. Digital photographs of all sections at the same magnification are recommended to be taken and saved for counts of annual opaque zones on the PC monitor and for re-ageing if necessary.

Two or three arms/lobes are visible from the core area of the otolith. The distal lobe is not found in sectioned otoliths which including the core in most Pacific bluefin tuna otoliths. Opaque zones are found in both the dorsal arm and ventral arm of the sectioned otolith. The ventral arm grows longer and straighter with one or two flection points. Opaque zones are more distinct in this ventral arm and are recommended for opaque zone counts (Fig. 6).

Identification of otolith opaque zones

Opaque zones are counted following methods outlined in other tuna ageing studies (Anonymous, 2002; Farley *et al.*, 2006). Opaque zones close to the core (ca. up to 10 years) are difficult to identify and counts should be based on zonation color and the distance between the core and each opaque zones. Early growth (ca. up to 2 years) of Pacific bluefin

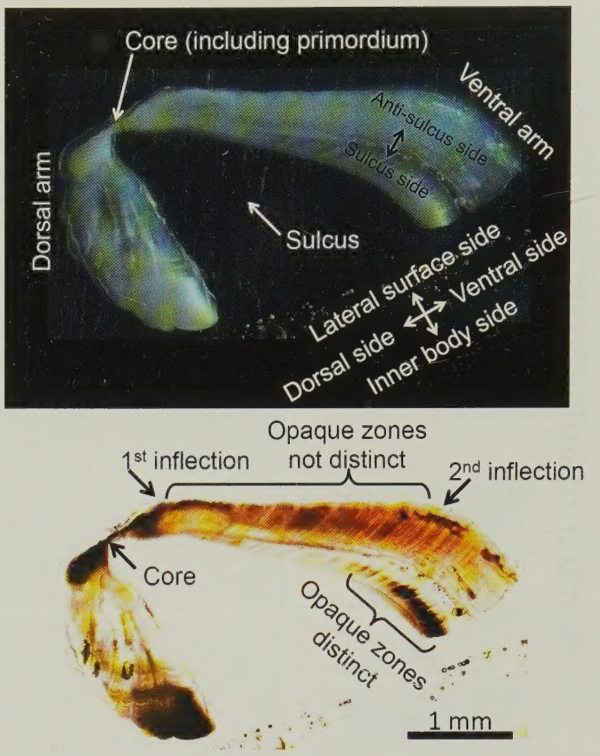


Fig. 6. Photographs of sectioned otolith of Pacific bluefin tuna *Thunnus orientalis* (243 cm FL, male). Reflected light (upper) and transmitted light (lower) show different images.

tuna has been accurately estimated by modal analysis of length frequency data and otolith micro-increment analysis, and fish are known to grow to 40–60 cm FL at age 1 year and 80–90 cm at two years (Yukinawa and Yabuta, 1967; Foreman, 1996). Using this information, individuals of 35–70 cm caught during April to June are thought to be one year old, and those of 70–95 cm are considered to be two years old (Fig. 7). As opaque zones form in April to July (see below) or even earlier for the first annual opaque zone (Shiao, unpublished data), the otoliths of individuals caught during these months are expected to possess one and two opaque zones, respectively.

Using these 1 and 2 year-old individuals, frequency distributions of the distances from the core to the middle of the 1st opaque zone and to the 2nd opaque zone were found to be unimodal (Fig. 8). Measurements from the core to the first opaque zone was 0.61 to 1.02 mm (n = 90, mean = 0.79), but it will vary depending on the hatch month of the individual fish because the peak spawning season

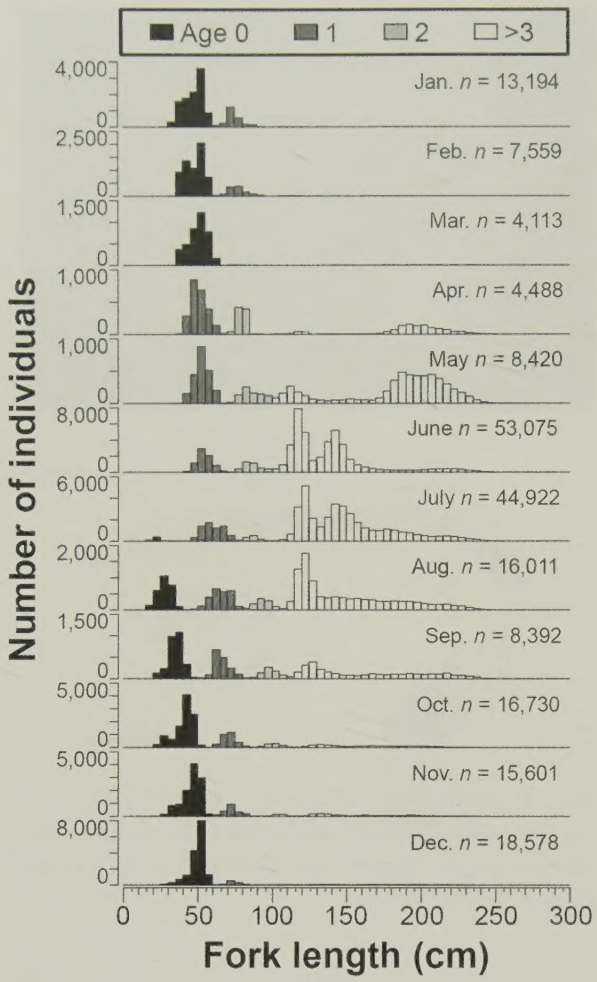


Fig. 7. Monthly changes in length frequency distribution of Pacific bluefin tuna *Thunnus orientalis*. Different age classes 0 to 2 years are recognized by their modes and plotted by different colors. Yearly age is assumed to increase on 1st April. Data are from RJB 2005 to 2010.

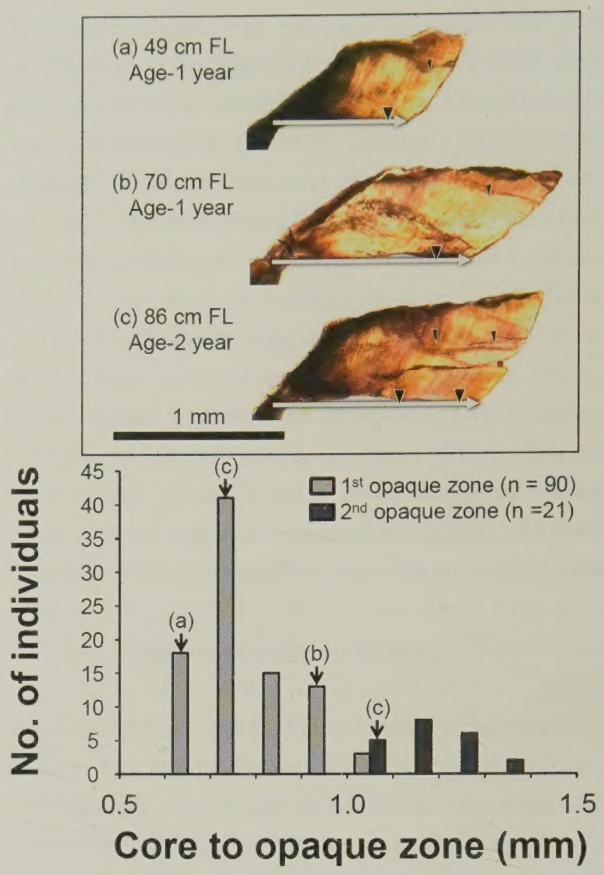


Fig. 8. Frequency distribution of distance from core to the 1st and the 2nd opaque zones of age 1 and 2 years Pacific bluefin tuna *Thunnus orientalis*. Measured parts of sectioned otolith from three different size fish are shown. (a) age-1 year small size (No. 2058, 49 cm FL, July), (b) age-1 year large size (No. 4898, 70 cm FL, July), (c) age-2 year (No. 4884, 86 cm FL, July). Arrows indicate recognized opaque zones.

extends from May to August (Itoh, 2009); i.e. early hatched fish (May-June) will have a longer distance from the core to the first opaque zone because they have more months to grow before the zone formed, while the late hatched fish (July-August) will have a shorter distance. The distance from the core to the middle of the second opaque zone was 1.07 to 1.39 mm (n = 21, mean = 1.18). These results provide useful criteria to identify the first and the second annual opaque zones.

The opaque zones between the first and second inflexion points are difficult to identify (i.e., the 1st to the ca. 9th opaque zones) (Fig. 6). The opaque zones form at similar distances apart, possibly because Pacific bluefin tuna grow almost linear until they reach ca. 200 cm FL (ca. 9 years old) (Shimose *et al.*, 2009), although measurements become slightly narrower as age increases. Opaque zones in this area should be counted referring to both the sulcus and anti-sulcus sides (Fig. 6).

After the second inflexion point, the opaque zones form uniformly and are more closely spaced. This is because the growth rate of Pacific bluefin tuna decreases after ca. 10 years old, but the otolith still increases in size. Opaque zones after the second inflection point should be counted on the sulcus side rather than the anti-sulcus side. Indistinct opaque zones (semi-annual opaque zones) are also formed in some otoliths even occurring in after the 10th opaque zones, but they are easily identified because they are

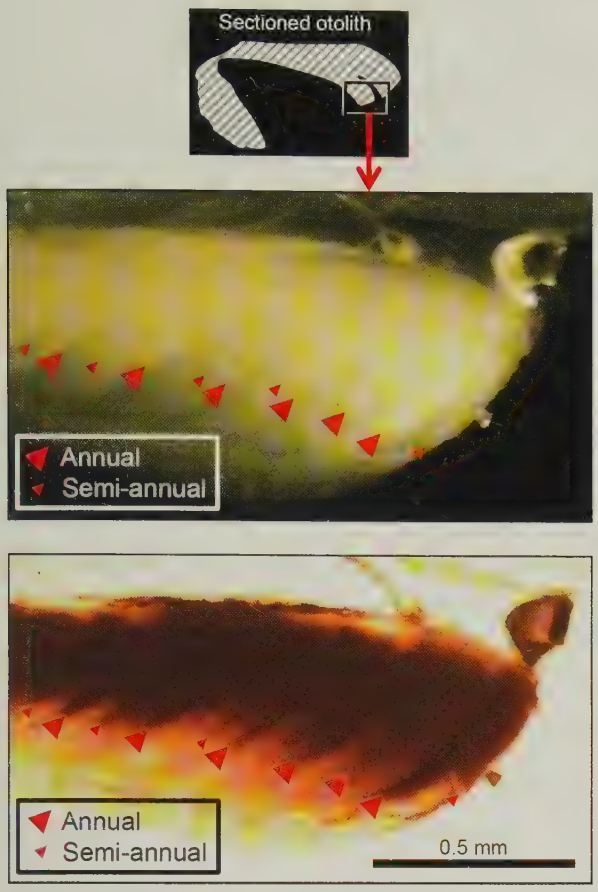


Fig. 9. Photographs of sectioned otolith near the edge of Pacific bluefin tuna *Thunnus orientalis* (No. 6236, 229 cm FL, October, Tsugaru Strait area) showing presumed annual and semi-annual (false) opaque zones. Reflected (upper) and transmitted (lower) light images are shown.

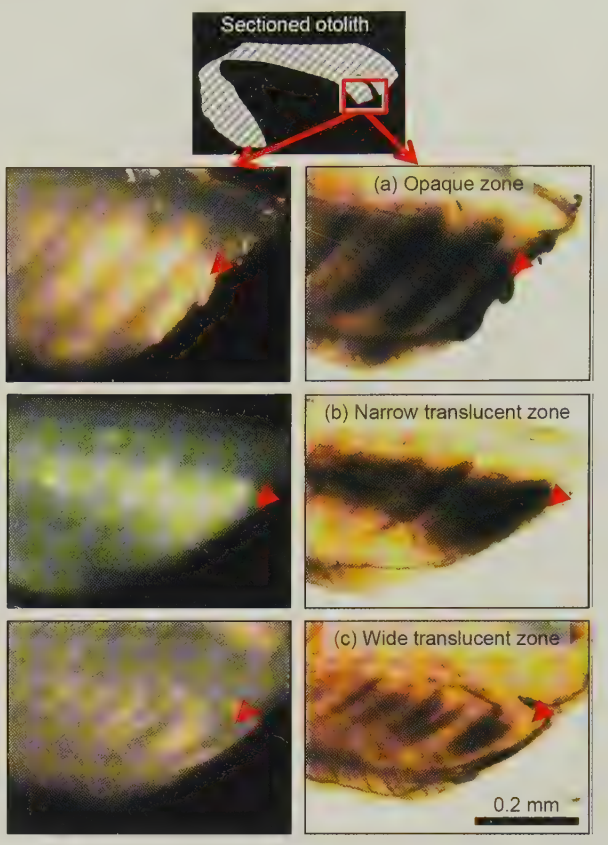


Fig. 10. Photographs of three types of edge condition on the sectioned otolith of Pacific bluefin tuna *Thunnus orientalis*. (a) Opaque zone (No. 5697, 206 cm FL, May, Nansei Islands area), (b) narrow translucent zone (No.6250, 187 cm FL, November, Tsugaru Strait area), (c) wide translucent zone (No. 5731, 200 cm FL, June, Nansei Islands area). Reflected (left) and transmitted (right) light images are shown.

faint and less prominent than true annual opaque zones (Fig. 9). These indistinct opaque zones tend to form around December, which is not consistent with the timing of typical annual opaque zones formation which occurs during April to July (Shimose *et al.*, 2009).

Records of age determination results

The number of opaque zones and edge condition are usually recorded for individual fish in age determination study. In the case of Pacific bluefin tuna, the readability score of each otolith is also recorded for possible re-examination or exclusion of low readability otoliths. All counts should be performed without any reference to fish length, locality or collection date. Name of reader and counting date/period are also recorded in each time. The followings are recommended items to record:

1) Number of opaque zones (include if an opaque zone is forming on the otolith edge): if there are multiple possible results, all of them should be recorded. This number does not necessarily represent the age of the fish.
2) Readability score: five degrees are recommended; 1) unreadable, 2) unsure with count, 3) slightly unsure in some zones, 4) confident with count, 5) very confident with no doubt in the result.
3) Edge condition: opaque zone or translucent zone. And if possible, subcategorized narrow or wide translucent zones (Fig. 10). Observation of the edge may be better using reflected light with black background and

higher magnification. Information about edge type is used for adjusting the individual age assignment (see below). Although the edge condition is difficult to identify in young individuals, it should be decided by the distance from the last counted opaque zone to otolith edge.

4) Note: other observations about sample condition or pattern characteristics; e.g. section broken, surface damaged, abnormal shape, indistinct (regarded as semi-annual) opaque zones found etc.

In the case of southern bluefin tuna, three otolith readings are recommended for each individual (Anonymous, 2002). If age determination is performed by a single reader, then two independent readings are recommended with an interval of at least 2 weeks between readings. If the counts differ, then a third reading is conducted to resolve to the final count. If there are two readers, the first and second counts are made by the two readers independently, and the final counts are conducted by both readers with access to their previous counts.

If possible, counted opaque zones are marked on the digital image of sectioned otolith to compare other counts and/or future reference. Currently, "Inkscape" software (http://www.inkscape.org/en/) is used to mark opaque zones in National Research Institute of Far Seas Fisheries.

Evaluation of precision on counts

Precision is a measure of the repeatability between multiple ageing results, and implies consistency (Campana, 2001; Anonymous, 2002). Precision of multiple counts is commonly evaluated by average percent error (APE; Beamish and Founier, 1981) and coefficient of variance (CV; Campana, 2001) in which a smaller values indicates better precision. These indices can be applied for multiple counts by a single reader or for multiple readers. The only available information on these values for Pacific bluefin tuna is 4.51 for mean APE and 6.38 for mean CV (within reader; Shimose *et al.*, 2009). These values are generally lower in larger individuals (Shimose *et al.*, 2009) because the opaque zones are more distinct in the older ages, reducing the proportion of miss-counts in the older fish. Current authors compared their counts between two readers for 100 specimens, and mean APE and CV were 4.88 and 6.90, respectively. Age-bias plots are also useful to compare counts between and among readers to determine if (and which age classes) bias is occurring (Campana, 2001).

Validation of otolith opaque zone formation

For southern bluefin tuna, the annual formation of opaque zones was validated directly and/or indirectly for both young and old individuals. Fifty nine southern bluefin tuna injected with strontium that were released and recaptured show that the 1st to 6th opaque zones are formed annually (Clear *et al.*, 2000). Bomb radiocarbon dating analysis showed that otolith core $\delta^{14}C$ values in old individuals was synchronized with the known values in seawater in the years when the southern bluefin tuna were hatched (as estimated by opaque zone counts) (Kalish *et al.*, 1996). This bomb radiocarbon dating analysis was also successfully applied to Atlantic bluefin tuna (Neilson and Campana, 2008).

Annual opaque zone formation is not validated for all age classes of Pacific bluefin tuna. Otolith edge condition was only identifiable in larger individuals for Pacific bluefin tuna (0.0 % for 46–99 cm FL, 18.2% for 100–149 cm, 55.8% for 150–199 cm, 94.1% for 200–260cm; Shimose *et al.*, 2009). Using edge type analysis, the formation of opaque zones in larger fish was validated to be annual (Shimose *et al.*, 2009). Opaque zones formed in smaller/younger fish are less distinct and have not been validated as annual yet. However, the early growth curve (<5 years) estimated using sectioned otolith (47–260 cm FL; Shimose *et al.*, 2009) is similar to the growth rates estimated by other reliable techniques (Fig. 11); e.g. tag-recapture analysis (Bayliff *et al.*, 1991) and otolith daily growth increment analysis (Foreman, 1996). Therefore, the age determination method using counts of opaque zones in sectioned otoliths is assumed to be possible for both younger and older individuals. Further direct validation studies are needed to obtain reliable length-at-age data.

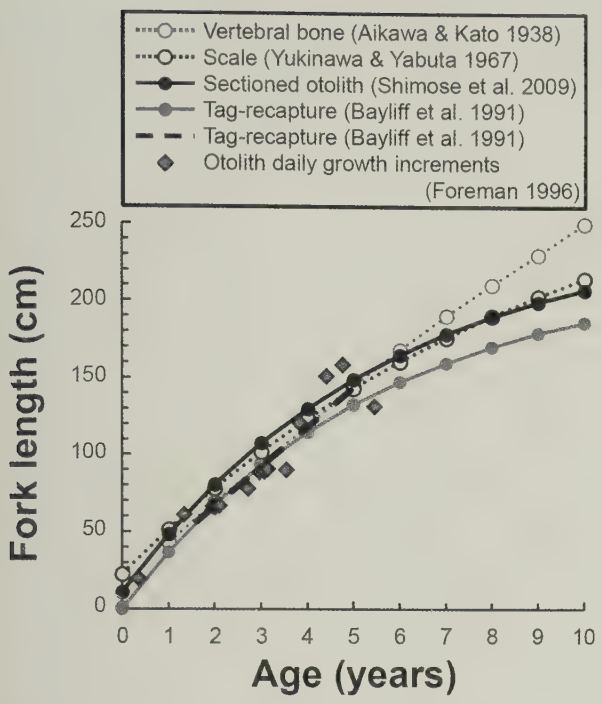


Fig. 11. Comparison in growth of Pacific bluefin tuna *Thunnus orientalis* estimated by various methods.

Assignment of age

Individual ages are estimated using the count of opaque zones, otolith edge condition, catch date, assumed spawning month, and timing of opaque zone formation (Campana, 2001). Spawning of Pacific bluefin tuna is known to occur from May to June in the southwestern North Pacific (Chen *et al.*, 2006; Itoh, 2009; Okochi, unpublished data) and from July to August in the Sea of Japan (Tanaka *et al.*, 2007; Itoh, 2009; Okochi, unpublished data). The former spawning group is thought to contribute more to the catch of age-0 Pacific bluefin tuna (Itoh, 2009), and the second quarter of the year (April to June) is assumed to be main spawning season (15 May, if the middle date is used). Otolith opaque zones of Pacific bluefin tuna form mainly during April to July corresponding to their spawning season at least for older individuals (Shimose *et al.*, 2009). As the spawning and opaque zone formation months are similar, the number of otolith opaque zones is almost equal to their annual age. However, true hatch (spawned) date is unknown and the opaque zone formation month may also different among individuals.

The following two adjustments are useful to correct for these variations;

1) Specimens with an opaque zone on otolith edge collected in the first quarter of the year (January–March) are aged as number of opaque zones −1 year. This is because they have a new opaque zone forming on the otolith edge before they truly reach the birth months (= the second quarter). This assumption is based on the information that main spawning season of Pacific bluefin tuna is May–June (nearly equal to the second quarter of the year, April–June).
2) Specimens with a wide translucent edge collected in the second quarter (April–June) are aged as number of opaque zones +1 year. This is because their new opaque zone is not formed yet despite they actually reach the birth months.

Although more adjustment may be needed, these two major adjustments are reasonably applicable and thought to be sufficient at this stage. Ages may be assigned as 0.25 year interval to produce a smoother growth curve (Shimose *et al.*, 2009); i.e. +0.25 years if caught in July to September, +0.50 years if caught in October to December, and +0.75 years if caught in January to March. Furthermore, birth date and/or recruitment month can be adjusted and fixed to fit the stock assessment model.

Reference collection

Reference collection images of sectioned otolith of Pacific bluefin tuna are prepared. There are two types of images; simple images of ventral arm area of sectioned otolith photographed using transmitted light, and the same image with marks showing opaque zones counted by authors. This collection consists of 90 individuals measured 50 to 260 cm FL from various fishing areas. Images of this collection will be available by request to authors (email: T.S.; shimose@affrc.go.jp or T.I.; ishiha@affrc.go.jp).

Conclusion

Age determination of Pacific bluefin tuna is improved by the development of the sectioned

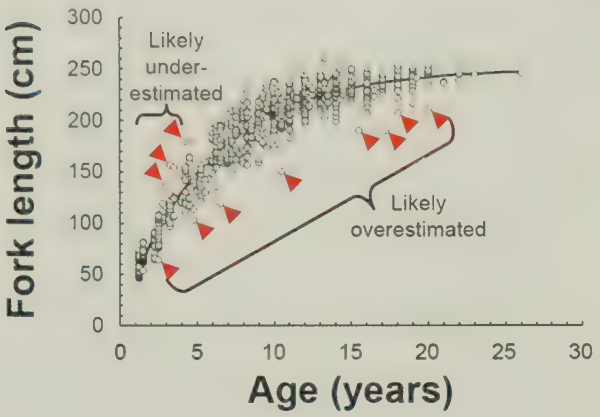


Fig. 12. Length at age and von Bertalanffy growth curve of Pacific bluefin tuna *Thunnus orientalis*. Modified from Shimose *et al.* (2009). Arrows show outlier individuals which may indicate possibly misinterpretation of ages.

otolith method, although the method appears to include some incorrect results (Fig. 12). Collecting more samples and accumulating data are necessary for increasing reliability of the current growth curve for Pacific bluefin tuna. Sex-specific growth and area specific age compositions are also needed and will require further studies.

The age determination manual for southern bluefin tuna "A manual for age determination of southern bluefin tuna *Thunnus maccoyii* (Anonymous, 2002)" may be useful when trying to determine the age of any other tuna species.

Acknowledgements

The authors are grateful to J.H. Farley (CSIRO, Australia), D. Gillespie (Fisheries and Oceans Canada), J. C. Shiao (National Taiwan University), R. J. D. Wells (National MarineFisheries Service, NOAA), O. Abe, Y. Takeuchi (National Research Institute of Far Seas Fisheries) for reviewing and comments to the earlier version of this manuscript.

Reference

Aikawa H., and Kato M., 1938: Age determination of fish. I. *Nippon Suisan Gakkaishi*, **7**, 79–88.

Anonymous, 2002: A manual for age determination of southern bluefin tuna, *Thunnus maccoyii*. Otolith sampling, preparation and interpretation. The Direct Age Estimation Workshop of the CCSBT 11–14 June, 2002, Queenscliff, Australia. 1–36 pp. http://www.ccsbt.org/docs/pdf/about_the_commission/age_determination_manual.pdf

Bayliff W. H., Ishizuka Y., and Deriso R. B., 1991: Growth, movement, and attrition of northern bluefin tuna, *Thunnus thynnus*, in the Pacific Ocean, as determined by tagging. *IATTC Bull.*, **20**, 1–94.

Beamish R. J., and Fournier D. A., 1981: A method for comparing the precision of a set of age determinations. *Can. J. Fish. Aquat. Sci.*, **38**, 982–983.

Campana S. E., 2001: Accuracy, precision and quality control in age determination, including a review of the use and abuse of age validation methods. *J. Fish Biol.*, **59**, 197–242.

Chen K. S., Crone P., and Hsu C. C., 2006: Reproductive biology of female Pacific bluefin tuna *Thunnus orientalis* from south-western North Pacific Ocean. *Fish. Sci.*, **72**, 985–994.

Clear N. P., Gunn J. S., and Rees A. J., 2000: Direct validation of annual increments in the otoliths of juvenile southern bluefin tuna, *Thunnus maccoyii*, by means of a large-scale mark-recapture experiment with strontium chloride. *Fish. Bull.*, **98**, 25–40.

Farley J. H., Clear N. P., Leroy B., Davis T. L. O., and McPherson G., 2006: Age, growthand preliminary estimates of maturity of bigeye tuna, *Thunnus obesus*, in the Australian region. *Mar. Freshw. Res.*, **57**, 713–724.

Foreman T., 1996: Estimates of age and growth, and an assessment of ageing techniques, for northern bluefin tuna, *Thunnus thynnus*, in the Pacific Ocean. *IATTC Bull.*, **21**, 75–123.

Gunn J. S., Clear N. P., Carter T. I., Rees A. J., Stanley C. A., Farley J. H., and Kalish J. M., 2008: Age and growth in southern bluefin tuna, *Thunnus maccoyii* (Castelnau): Direct estimation from otoliths, scales and vertebrae. *Fish. Res.*, **92**, 207–220.

Itoh T., 2009: Contributions of different spawning seasons to the stock of Pacific bluefin tuna *Thunnus orientalis* estimated from otolith daily increments and catch-at-length data of age-0

fish. *Nippon Suisan Gakkaishi*, **75**, 412–418.

Kalish J. M., Johnston J. M., Gunn J. S., and Clear N. P., 1996: Use of the bomb radiocarbon chronometer to determine age of southern bluefin tuna *Thunnus maccoyii*. *Mar. Ecol. Prog. Ser.*, **143**, 1–8.

Neilson J. D. and Campana S. E., 2008: A validated description of age and growth of western Atlantic bluefin tuna (*Thunnus thynnus*). *Can. J. Fish. Aquat. Sci.*, **65**, 1523–1527.

Shimose T., Tanabe T., Kai M., Muto F., Yamasaki I., Abe M., Chen K. S., and Hsu C. C., 2008: Age and growth of Pacific bluefin tuna, *Thunnus orientalis*, validated by the sectioned otolith ring counts. *ISC/08/PBF/01/08*.

Shimose T., Tanabe T., Chen K. S., and Hsu C. C., 2009: Age determination and growth of Pacific bluefin tuna, *Thunnus orientalis*, off Japan and Taiwan. *Fish. Res.*, **100**, 134–139.

Tanabe T. and Kai M., 2007: Estimates of the age-length relationship of Pacific bluefin tuna with descriptive analysis on uncertainties remained. *ISC/07/PBFWG-2/03*.

Tanaka Y., Mohri M., and Yamada H., 2007: Distribution, growth and hatch date of juvenile Pacific bluefin tuna *Thunnus orientalis* in the coastal area of the Sea of Japan. *Fish. Sci.*, **73**, 534–542.

Yukinawa M., and Yabuta Y., 1967: Age and growth of the bluefin tuna, *Thunnus thynnus* (Linnaeus), in the North Pacific Ocean. Rep. *Nankai Reg. Fish. Res. Lab.*, **25**, 1–18.

Preface

The 41st Joint Meeting of the UJNR Aquaculture Panel was conducted from October 2013 (October 9th – 13th at Sapporo and Hakodate, Hokkaido) through December 2013 (December 10th – 13th at Yokohama, Kanagawa and Miyako, Iwate). Through the long history of UJNR, Aquaculture Panel has contributed to development of aquaculture researches of both countries by means of various cooperative activities, i.e. the exchange of scientists, the exchange of literatures and promoting joint research projects. It is understood that the Aquaculture Panel is one of the most active UJNR Panels by both countries. Further positive action is expected to the UJNR Aquaculture Panel for a solution of the varied problems presenting in aquaculture of both countries.

The present special issue of Bulletin of Fisheries Research Agency is the proceedings of the 41st UJNR Aquaculture Panel Symposium "Advanced Aquaculture Technologies". This Symposium was a venue to exchange original, high-quality research on new and developing technologies that give promise to improve or revolutionize aquaculture, based on the key bottleneck issues identified at the 39th Scientific Symposium "The Present and Future of the Aquaculture Industry".

Further development of aquaculture is highly desired as a production source of important foods for human being all over the world. Stable supplies of safe and economical feed ingredients are requisite for the sustainable development of aquaculture. Nevertheless, in recent years we recognized some serious problems, which threatened the use of important feed ingredients like fish meal and oil. It is my great pleasure that the present UJNR proceedings containing high quality papers of the selected American and Japanese aquaculture scientists will help integrating aquaculture and fisheries technologies to optimize value from coastal resources, zoning for aquaculture, use of biotechnology in aquaculture and effects on natural population, and improvement of public perception.

Finally I would like to express my sincere gratitude to the colleagues involved in the UJNR Aquaculture Panel for their efforts to prepare and organize the symposium. I also would like to deeply thank the editorial board members for publishing the proceedings.

Fuminari Ito
Chair of UJNR Aquaculture Panel
Executive Director
Incorporated Administrative Agency,
Fisheries Research Agency

CONTENTS

Preface
Contents
Program

Overview of Aquaculture in the US and Japan

1. The Fisheries Agency's policy directions for aquaculture
Kazumasa Ikuta (Counselor, Resource Enhancement Promotion Department Japan Fisheries Agency, Ministry of Agriculture Forestry and Fisheries) ····· 19
2. Aquaculture in the US: The first annual national report on aquaculture
Marie Fujitani (NOAA Office of Aquaculture) ····· 21
3. The challenge of reconstructing coho salmon aquaculture after the Great East Japan Earthquake and Tsunami in 2011
Ikutaro Shimizu (National Research Institute of Fisheries Science, FRA)····· 33

Fish Diet and Nutrition

4. Development of a new type of fish diet, non-fish meal extruded-pellet
Noriko Ishida (National Research Institute of Fisheries Science, FRA) ····· 39
5. The importance of taurine and n-3 fatty acids in cobia nutrition
Aaron Watson (University of Maryland) ····· 51
6. Availability of fisheries by-product materials with cadmium removal treatment as a feed ingredient for fingerling black rockfish *Sebastes schlegeli*
Nobukazu Satoh (Fisheries Research Department Mariculture Fisheries Research Institute, Hokkaido Research Organization) ····· 61
7. Development and characterization of several open formula reference diets for marine fish larvae
Ken Webb (NOAA, Northwest Fisheries Science Center) ····· 67
8. Effect of feed ingredients on digestive enzyme secretion in fish
Koji Murashita (National Research Institute of Aquaculture, FRA) ····· 69
9. Cholecystokinin and trypsin responses of larval red drum (*Sciaenops ocellatus*) in response to algae, live prey, and inert particles
Michael B. Rust (NOAA) ····· 75

Management, Social and Economic Issues of the Aquaculture Industry

10. An analysis of the causality between the market price of imported fishmeal and market price of marine farmed fish
Yoshifumi Takahashi (Tohoku National Fisheries Research Institute, FRA) ····· 79
11. Good aquaculture practices to reduce the use of chemotherapeutic agents, minimize bacterial resistance, and control product quality
Stanley Serfling (Food and Drug Administration Office of Food Safety) ····· 83
12. Modeling intraspecific genetic effects for management of aquaculture programs
Jason Volk (ICF International) ····· 89

Techniques of Aquaculture Production

13. Specific monovalent and divalent ion supplementation for culture of marine species
Chris Green (Louisiana State University Agricultural Center) ····· 97

14. Genetically modified salmon in aquaculture: well regulated and safe
Paul G. Olin (UC San Diego/ Scripps Institution of Oceanography) ····· 109

15. Reproductive dysfunction in cultured sablefish (*Anoplopoma fimbria*)
Jose Guzman (NOAA) ····· 111

Invertebrate Aquaculture I: Echinoderm

16. Induced spawning in the sea cucumber *Apostichopus japonicus* by neuropeptide, cubifrin
Keisuke Yamano (National Research Institute of Aquaculture, FRA) ····· 121

17. Mass production of artificial seed of the Japanese common sea cucumber (*Apostichopus japonicus*) in Hokkaido, Japan
Yuichi Sakai (Fisheries Research Department Mariculture Fisheries Research Institute, Hokkaido Research Organization) ····· 129

18. Fluorochrome marking of out-planted green sea urchins, *Strongylocentrotus droebachiensis*, for sea ranching and restocking programs in the Gulf of Maine, USA
Stephen Eddy (University of Maine, Center for Cooperative Aquaculture Research) ····· 135

19. Improving the food quality of sea urchin gonads by suppressing gametogenesis
Tatsuya Unuma (Hokkaido National Fisheries Research Institute, FRA) ····· 145

20. Maturation control of the short-spined sea urchin, *Strongylocentrotus intermedius*, by low temperature rearing using deep-sea water, with the aim of extending the market season
Takaaki Kayaba (Kushiro Fisheries Research Institute, Hokkaido Research Organization) ····· 155

Invertebrate Aquaculture II: Mollusc

21. Long-term outcomes in the tech transfer of scallop spat collection techniques, from Aomori Prefecture, Japan to Maine, USA
Dana Morse (Maine Sea Grant) ····· 167

22. Oyster culture in Hokkaido, Japan
Natsuki Hasegawa (National Research Institute of Aquaculture, FRA)····· 173

The 41st Scientific Symposium of the UJNR Aquaculture Panel

October 9th – 10th, 2013

Advanced Aquaculture Technologies

Program

Wednesday, October 9th, 2013 Registration 13 : 00–17 : 00

Welcome and opening remarks (Masaaki Fukuda, Director,
Hokkaido National Fisheries Research Institute, FRA) ····· 13 : 30–13 : 45
Aim of the Symposium (Katsuma Hanafusa, Japan Panel Chair) ····· 13 : 45–14 : 00

Session Ⅰ. Overview of Aquaculture in the US and Japan

(Moderators: P. Olin & Y. Machiguchi)

1. The Fisheries Agency's policy directions for aquaculture
   Kazumasa Ikuta (Counselor, Resource Enhancement Promotion Department
   Japan Fisheries Agency, Ministry of Agriculture Forestry and Fisheries)
   ····· 14 : 00–14 : 20
2. Aquaculture in the US: The first annual national report on aquaculture
   Marie Fujitani (NOAA Office of Aquaculture) ····· 14 : 20–14 : 40
3. The challenge of reconstructing coho salmon aquaculture after the
   Great East Japan Earthquake and Tsunami in 2011
   Ikutaro Shimizu (National Research Institute of Fisheries Science, FRA)
   ····· 14 : 40–15 : 00

Discussion ····· 15 : 00–15 : 10

Break ····· 15 : 10–15 : 20

Session Ⅱ. Fish Diet and Nutrition

(Moderators: K. Webb & K. Murashita)

4. Development of a new type of fish diet, non-fish meal extruded-pellet
   Noriko Ishida (National Research Institute of Fisheries Science, FRA)
   ····· 15 : 20–15 : 40
5. The importance of taurine and n-3 fatty acids in cobia nutrition
   Aaron Watson (University of Maryland) ····· 15 : 40–16 : 00
6. Availability of fisheries by-product materials with cadmium removal
   treatment as a feed ingredient for fingerling black rockfish *Sebastes schlegeli*
   Nobukazu Satoh (Fisheries Research Department Mariculture
   Fisheries Research Institute, Hokkaido Research Organization) ····· 16 : 00–16 : 20
7. Development and characterization of several open formula reference
   diets for marine fish larvae
   Ken Webb (NOAA, Northwest Fisheries Science Center) ····· 16 : 20–16 : 40

8. Effects of diets supplemented with herb essential oils on ectoparasitic protozoan infections on chum salmon (*Oncorhynchus keta*) fry
Shinya Mizuno (Salmon and Freshwater Fisheries Research Institute, Hokkaido Research Organization) ····· 16 : 40-17 : 00

Discussion ····· 17 : 00-17 : 20

Thursday, October 10th, 2013 Registration 9 : 00- 9 : 20

Session Ⅱ. Fish Diet and Nutrition; continued
(Moderators: A. Watson & N. Satoh)

9. Effect of feed ingredients on digestive enzyme secretion in fish
Koji Murashita (National Research Institute of Aquaculture, FRA) ··· 9 : 20- 9 : 40
10. Cholecystokinin and trypsin responses of larval red drum (*Sciaenops ocellatus*) in response to algae, live prey, and inert particles
Michael B. Rust (NOAA) (Presented by Ken Webb) ····· 9 : 40-10 : 00

Discussion ····· 10 : 00-10 : 10

Session Ⅲ. Management, Social and Economic Issues of the Aquaculture Industry
(Moderators: M. Fujitani & I. Shimizu)

11. An analysis of the causality between the market price of imported fishmeal and market price of marine farmed fish
Yoshifumi Takahashi (Tohoku National Fisheries Research Institute, FRA) ····· 10 : 10-10 : 30
12. Good aquaculture practices to reduce the use of chemotherapeutic agents, minimize bacterial resistance, and control product quality
Stanley Serfling (Food and Drug Administration Office of Food Safety) (Presented by M. Fujitani) ····· 10 : 30-10 : 50
13. Modeling intraspecific genetic effects for management of aquaculture programs
Jason Volk (ICF International) ····· 10 : 50-11 : 10

Discussion ····· 11 : 10-11 : 20

Group Photo Session ····· 11 : 20-11 : 40

Lunch Break ····· 11 : 40-13 : 00

Session Ⅳ. Techniques of Aquaculture Production
(Moderators: J. Volk & K. Okuzawa)

14. Specific monovalent and divalent ion supplementation for culture of marine species
Chris Green (Louisiana State University Agricultural Center) ····· 13 : 00-13 : 20
15. Genetically modified salmon in aquaculture: well regulated and safe
Paul G. Olin (UC San Diego/ Scripps Institution of Oceanography) ··· 13 : 20-13 : 40
16. Reproductive dysfunction in cultured sablefish (*Anoplopoma fimbria*)
Jose Guzman (NOAA) ····· 13 : 40-14 : 00

Discussion ····· 14 : 00–14 : 10

Session V. Invertebrate Aquaculture I: Echinoderm

(Moderators: S. Eddy & T. Unuma)

17. Induced spawning in the sea cucumber *Apostichopus japonicus* by neuropeptide, cubifrin
    Keisuke Yamano (National Research Institute of Aquaculture, FRA)
    ····· 14 : 10–14 : 30
18. Mass production of artificial seed of the Japanese common sea cucumber (*Apostichopus japonicus*) in Hokkaido, Japan
    Yuichi Sakai (Fisheries Research Department Mariculture Fisheries Research Institute, Hokkaido Research Organization) ····· 14 : 30–14 : 50

Break ····· 14 : 50–15 : 00

(Moderators: D. Morse & K. Yamano)

19. Fluorochrome marking of out-planted green sea urchins, *Strongylocentrotus droebachiensis*, for sea ranching and restocking programs in the Gulf of Maine, USA
    Stephen Eddy (University of Maine, Center for Cooperative Aquaculture Research) ····· 15 : 00–15 : 20
20. Improving the food quality of sea urchin gonads by suppressing gametogenesis
    Tatsuya Unuma (Hokkaido National Fisheries Research Institute, FRA)
    ····· 15 : 20–15 : 40
21. Maturation control of the short-spined sea urchin, *Strongylocentrotus intermedius*, by low temperature rearing using deep-sea water, with the aim of extending the market season
    Takaaki Kayaba (Kushiro Fisheries Research Institute, Hokkaido Research Organization) ····· 15 : 40–16 : 00

Session VI. Invertebrate Aquaculture II: Mollusc

(Moderators: C. Green & Y. Sakai)

22. Long-term outcomes in the tech transfer of scallop spat collection techniques, from Aomori Prefecture, Japan to Maine, USA
    Dana Morse (Maine Sea Grant) ····· 16 : 00–16 : 20
23. Relationship between spat density and environmental factors in cultured Japanese Scallop, *Mizuhopecten yessoensis* in Funka Bay, Japan.
    Katsuhisa Babà (Hakodate Fisheries Research Institute, Hokkaido Research Organization) ····· 16 : 20–16 : 40
24. Oyster culture in Hokkaido, Japan
    Natsuki Hasegawa (National Research Institute of Aquaculture, FRA)
    ····· 16 : 40–17 : 00

Discussion ····· 17 : 00–17 : 10

Closing remarks (Paul Olin, U.S.A. Panel Chair) ····· 17 : 10–17 : 20

水研センター研報，第40号，19，平成27年
Bull. Fish. Res. Agen. No. 40, 19, 2015

The Fisheries Agency's Policy Directions for Aquaculture

Kazumasa IKUTA*1

Abstract: Aquaculture is an important industry in Japan, and production levels occupy approximately 20% in terms of yield, and 30% in terms of market value, of the country's total fisheries production. However, the economic aspects of aquaculture have been precarious due to the low price of certain products and increasing costs of production. In addition, the guarantee of food safety, minimization of environmental impact, and management of natural stock populations are highly necessitated in order to achieve the sustainability of the industry.

In order to address these problems, the Fisheries Agency held a series of committee meetings during 2013 in order to develop ideal projections for the aquaculture industry. Thereafter, policy directions for aquaculture were put forth as follows:

1) Measures to improve unstable business practices of aquaculture should be implemented; this includes the expansion of mutual-aid systems, conversion of feed resources to alternative usages of protein, maintenance of carrying capacity, improvement of systems to compensate for increases in feed costs, and the promotion of planned production including the enhanced exportation of products.
2) Measures to improve production techniques should be implemented; this includes achieving improved food safety based on production systems that have high traceability, risk management and the usage of vaccines, conservation of the environment in and around aquaculture grounds, a shift from the use of wild fish seedlings and natural feed resources to the use of artificially-produced seedlings and feeds, the development of enclosed recirculating aquaculture systems, and R&D for advanced aquaculture technologies that will reduce costs and enhance productivity such as offshore aquaculture systems, enhanced breeding methodologies, and raft aquaculture systems for new bivalve species.

2015年1月30日受理（Received on January 30, 2015）
*1 Counselor, Resource Enhancement Promotion Department, Japan Fisheries Agency, Ministry of Agriculture Forestry and Fisheries
E-mail: ikutak@affrc.go.jp

水研センター研報，第40号，21－31，平成27年
Bull. Fish. Res. Agen. No. 40, 21－31, 2015

First Annual Report on Aquaculture and Policy in the United States of America for the 2013 UJNR Panel

Marie L. Fujitani*, Robert C. Jones*, and Mike B. Rust*

Abstract: The first U.S. National Report on Aquaculture for the 41st U.S. Japan Natural Resources Panel on Aquaculture overviews major policy items, current trends in aquaculture production, and other important developments affecting contemporary U.S. aquaculture.

Policy- April 2013 saw the release of the National Ocean Policy Implementation Plan (NOP-IP), a document to translate President Obama's 2010 National Ocean Policy (Executive Order 13547 --Stewardship of the Ocean, Our Coasts, and the Great Lakes) to specific federal actions. Aquaculture featured prominently in the Implementation Plan. The Joint Sub-committee on Aquaculture (JSA) was re-named the Interagency Working Group on Aquaculture (IWG-A), and was tasked with identifying and supporting milestones in the NOP-IP. The NOP-IP also supported the National Shellfish Initiative to increase shellfish production and restoration in U.S. waters. NOAA and the Gulf of Mexico Fishery Management Council are working on a Fishery Management Plan to permit aquaculture in federal waters in the Gulf of Mexico. The U.S. currently does not have a framework to permit aquaculture in the EEZ, and this would be a first for the U.S. if it goes into effect.

Developments- For the first time in some years, in 2013 many new sites for shellfish aquaculture have been permitted. This is attributed to increased interest in shellfish aquaculture, particularly in the northwest and northeast, federal interagency and state efforts on behalf of the Washington Shellfish Initiative, and state-level successes at streamlining permitting. In September 2013, the California Shellfish Initiative was launched. A program is underway to permit and restore Hawaiian fishponds, a form of traditional Hawaiian aquaculture, for cultural heritage, subsistence, and possibly commercial production.

Production Trends- Aquaculture production in the U.S. is largely composed of catfish, crawfish, trout, salmon, oysters, mussels, clams, tilapia, and shrimp. For the 15-year period from 1996–2011, the value of U.S. aquaculture showed 3% average year-over-year growth, though total volume fell by an average of 0.5%. Mariculture (salmon and shellfish), on the other hand, showed robust 4% by volume and 5% by value year-over- year average growth in the same period. 2011 is the most current year published aquaculture statistics are available. From 2010 to 2011, total U.S. aquaculture went down -18.8% by volume (to 277,335 metric tons) but grew 4.2% by value (to 1.34billion dollars). From 2010 to 2011 mariculture decreased by -12.4% by volume (to 35,739 metric tons) while growing 0.3% by value (to 314 million dollars). However, in the previous year from 2009–2010, mariculture grew 21.6% by volume (to 40,823 metric tons) and 28.5% by value (to 312 million dollars). Most of the growth in value for marine aquaculture was from salmon (*Salmo salar*) and oyster (mainly *Crassostrea gigas* and *Crassotrea virginica*) production.

Keywords: aquaculture policy, aquaculture production, annual report

2015年1月30日受理（Received on January 30, 2015）
* NOAA Office of Aquaculture, 1315 East-West Highway, Silver Spring, MD 20910
E-mail: NMFS.aquaculture.science@noaa.gov

Background

The U.S. imports over 90 percent of its seafood, half of which is a product of foreign aquaculture. While aquaculture produces 50 percent of all seafood eaten worldwide, in 2011 U.S. domestic aquaculture production was only 9% of total domestic landings. Total domestic aquaculture in the U.S. provided around 5% of the seafood supply; mariculture was the source of less than 1% of U.S. seafood.

Policy

The Department of Commerce, NOAA Fisheries, and Aquaculture: Marine aquaculture is an important part of the missions of the Department of Commerce (DOC) and the National Oceanic and Atmospheric Administration (NOAA). The DOC sees aquaculture as a way to create jobs and economic activity. A federal agency under the DOC, NOAA sees aquaculture as a critical component to meeting increasing global demand for seafood and maintaining healthy ecosystems.

The NOAA Office of Aquaculture is within the National Marine Fisheries Service (NMFS), a branch of NOAA. NMFS is involved in aquaculture from both science and policy perspectives: science to foster efficiency and sustainability and policy to enable marine aquaculture while ensuring environmental responsibility. Marine aquaculture is an increasingly important part of the NOAA NMFS mission. Marine aquaculture is providing a growing amount of seafood to U.S. consumers, and supports commercial and recreational fisheries and helps restore species and habitat.

Aquaculture is garnering increasing attention from Commerce, members of Congress, and the private business sector for its ability to provide economic opportunities (especially in fishing communities) and potential to grow more seafood domestically in a safe, sustainable way. In 2011, NOAA and the Department of Commerce released complimentary aquaculture policies (http://www.nmfs.noaa.gov/aquaculture/policy/2011_policies_homepage.html) supporting aquaculture to enable domestic production of seafood, maintain and restore healthy marine ecosystems, and create employment and business opportunities.

The National Ocean Policy Implementation Plan: In 2010 President Obama signed the National Ocean Policy (NOP; Executive Order 13547 --Stewardship of the Ocean, Our Coasts, and the Great Lakes, http://www.whitehouse.gov/the-press-office/executive-order-stewardship-ocean-our-coasts-and-great-lakes). In April 2013 the National Ocean Policy Implementation Plan (NOP-IP, http://www.whitehouse.gov/administration/eop/oceans/implementationplan) was released, which charges federal agencies to work together and take specific actions to address ocean issues of national importance. Aquaculture is featured prominently in the Implementation Plan.

The aquaculture-specific actions in the NOP-IP relate to increasing efficiencies in the aquaculture permitting processes, coordinating agency participation, and providing jobs and economic value by protecting and restoring coastal wetlands, coral reefs, and other natural systems. Along with goals for commercial aquaculture, restoration is also specifically mentioned in the NOP-IP, to improve coastal and estuarine restoration efforts through better monitoring, coordination, and planning.

Agency Abbreviations:
NOAA: National Oceanic and Atmospheric Administration
USDA: U.S. Department of Agriculture
ARS: Agricultural Research Service
NIFA: National Institute of Food and Agriculture
APHIS: Animal and Plant Health Inspection Service
EPA: Environmental Protection Agency
USCG: U.S. Coast Guard
DOC: Department of Commerce
DOI: Department of the Interior
DOL: Department of Labor
USFWS: U.S. Fish and Wildlife Service
FERC: Federal Energy Regulatory Commission
DOE: Department of Energy
HHS: Department of Health and Human Services
FDA: Food and Drug Administration
ACOE: The U.S. Army Corps of Engineers

NOP-IP Actions Relating to Aquaculture, and Responsible Agencies:

By the end of 2013

- Develop and implement permitting regulatory efficiencies for aquaculture. [NOAA, USDA, EPA, USACE, USCG, DOI (USFWS)]

- Establish an interagency aquaculture initiative that supports jobs and innovation through the National Science and Technology Council's Interagency Working Group on Aquaculture and other partnerships. [DOC, NOAA, USDA (ARS, NIFA)]
- Through leveraging existing research priorities, provide scientific information on the environmental health effects of finfish aquaculture to streamline permitting and improve water quality monitoring. [Aquaculture Regulatory Task Force]
- Through the National Shellfish Initiative develop pilot projects to identify ways to both maxi-mize the environmental sustainability and ecosystem benefits (e.g., nutrient filtration, carbon sequestration, fish habitat) and the commercial value of shellfish aquaculture. This would help develop a comprehensive plan to sustainably increase shellfish production and restore populations in U.S. waters. [NOAA, USDA (ARS, NIFA)]

By the end of 2015

- Identify and make available best management practices to inform and improve Federal permitting processes for aquaculture. [NOAA, USDA, EPA, USACE, USCG, DOI (USFWS)]
- Develop an analysis of the contribution and impacts (including job creation) of emerging uses—including renewable energy, aquaculture, and biotechnology—on the economies of the communities and regions dependent on marine and coastal resources. [NOAA, DOE, DOI, FERC, DOL, DOC]

By the end of 2016

- Develop and incorporate adaptation strategies for coastal and ocean species and habitats into future planning and management processes, such as fisheries, protected species, coral reefs, or shellfish aquaculture. [NOAA, DOI, EPA]

The Inter-Agency Working Group on Aquaculture (IWG-A): The Inter-agency Working Group on Aquaculture (IWG-A; formerly called the Joint Sub-committee on Aquaculture) is an inter-agency group under the auspices of the Life Sciences Sub-committee of the National Science and Technology council. The purpose of the working group is to increase the overall effectiveness and productivity of Federal aquaculture research, technology transfer, and technology assistance programs. Efficient, coordinated permitting processes will allow ocean industries, including commercial shellfish and finfish aquaculture, to save time and money and encourage economic growth without compromising federal agency responsibilities to protect health, safety, and the environment. Improved interagency coordination and reduced redundancy will decrease administrative waste, the burden on federal agencies, and the regulatory burden for industry.

Under the IWG-A, an Aquaculture Regulatory Task Force (hereafter 'task force') was created in 2013under the auspices of the National Aquaculture act of 1980, and will address specific milestones and activities in support of the Implementation Plan of the National Ocean Policy in consultation and partnership with the National Ocean Council and the IWG-A. The task force consists of the following federal agencies: The USDA: ARS, NIFA, and APHIS; DOC: NOAA; HHS: FDA; DOI: USFWS; EPA; and the ACOE. The task force also includes the following organizations in the Executive Office of the President: National Ocean Council;Office of Management and Budget; and Office of Science and Technology Policy. The task force will operate from 2013-2015 unless renewed.

In 2013 the task force assembled a draft charter to support aquaculture milestones in the NOP-IP, as well as a draft work plan identifying specific actions towards task force goals. Progress has been made on facilitating inter-agency communication, coordination to streamline multi-agency permitting processes, and increasing the ease of access to information about the permitting process.

The Gulf of Mexico Fishery Management Plan: The U.S. currently does not have a framework to permit long-term aquaculture projects in the Exclusive Economic Zone (federal waters), 3 to 200 miles (5 to 322 kilometers) offshore. This is despite the huge potential for offshore aquaculture in the U.S. A recent publication from the Food and Agricultural Organization (FAO) of the United Nations (Kapetsky *et al.*, 2013) listed the US as number one in the world for offshore marine production potential. Japan also

ranked highly (fifth) in this category. The rankings were obtained by accounting for the area in each country's Exclusive Economic Zone with appropriate depth, currents, temperature, and distance to ports for finfish (e.g. salmon and cobia) and shellfish (e.g. mussels) production.

An ongoing development for U.S. offshore aquaculture is the Fishery Management Plan for Regulating Offshore Marine Aquaculture in the Gulf of Mexico (Aquaculture FMP). The Gulf of Mexico Fishery Management Council proposed regulations for an Aquaculture FMP, the first time that a regional fishery management council has approved a comprehensive regulatory program for offshore aquaculture in U.S. federal waters. NOAA is currently reviewing the proposed regulations that would implement the Aquaculture FMP. The Fishery Management Plan authorizes NOAA Fisheries to issue permits to culture species managed by the Gulf of Mexico Fishery Management Council, with the exception of shrimp and corals. We expect final regulations to be effective sometime in 2014.

Recent Key Developments in U.S. Aquaculture

National and Regional Shellfish Initiatives: The National Shellfish Initiative is a policy created in conjunction with the 2011 DOC and NOAA Aquaculture Policies. The goal of the initiative is to support increases in shellfish production and restoration in U.S. waters. In 2013 the Office of Aquaculture and aquaculture regional coordinators supported a number of actions for the National Shellfish Initiative in partnership with other NOAA offices, industry, the restoration community, and state and tribal partners in California, Washington State, the Northeast, and Hawaii.

The Washington State Shellfish Initiative, through the actions of the Washington State Shellfish Initiative Inter-agency Working Group, completed several products to streamline the site permitting process. Due to many factors (for example regulatory complexities and social license), for a number of years there have been difficulties with permitting new sites for shellfish aquaculture in some regions of the United States, particularly in the Pacific Northwest. Actions by the Washington State Shellfish Initiative Inter-agency Working Group to coordinate and streamline the permitting process in that state led to 7 new shellfish site permits, the first issued by the ACOE in six years.

The Washington State Shellfish Initiative also led to the Ocean Acidification Blue Ribbon panel report and new funding from the State of Washington for shellfish ocean acidification research. The Blue Ribbon Panel gave a summary of ocean acidification research, and a summary of recommendations for shellfish hatcheries and growers. Ocean acidification is of major concern on the west coast, particularly in the Pacific northwest. In previous years before ocean acidification was identified as the culprit and mitigation measures were employed, some hatcheries experienced up to 80% larval mortality. Research led to the finding that acidic water from upwelling put stress on larval shellfish, rendering them unable to build shells in a critical point in their development. Hatcheries now have sensors and only draw seawater when the pH level is 'safe' for larvae. However, this does not help with shellfish in the wild, and some bays have not seen a natural set in almost ten years. The Washington Shellfish initiative also led to funding construction of a hatchery in Manchester, WA, for native Olympia oyster seed.

In September 2013, the California Shellfish Initiative was launched with a kickoff meeting involving agencies and stakeholders to identify key actions for expanded shellfish farming and restoration to support and conserve California's unique ecosystem. Over 70 people attended. One outcome of this meeting was coordination between NMFS staff and the Humboldt Harbor District on shellfish aquaculture in Humboldt Bay, California. The Harbor District is pursuing an innovative approach for permitting, where the Harbor District owns a regional permit for the entire bay, bears the cost of the permitting process, and then issues leases to individual growers. This will allow small businesses to participate in shellfish aquaculture, because the permitting process can be too expensive and uncertain for a small business to navigate alone.

The Northeast U.S. has been very successful in streamlining the permitting process, and is seeing impressive growth in the shellfish sector. For example, over the last two years in Maryland, 1,100

acres have been leased for shellfish aquaculture, and 1,500 more acres are in the process. Over the same period in Virginia, over 5,000 oyster acres were permitted. The New England region (Maine, Vermont, New Hampshire, Massachusetts, Connecticut, and Rhode Island) is working on a general regional permit to streamline the permitting process for new shellfish aquaculture operations. In the state of Maine, state agencies and the ACOE are also pursuing a streamlined permitting system. Further, due to economic and fishery conditions, there are a growing number of fishermen and fishing families in the Northeast who are starting aquaculture in addition to or instead of fishing.

In Hawaii there is growing interest in raising oysters. The Hawaii State Department of Health just developed a process to certify waters to raise shellfish. The growth rate for oysters in Hawaii is high, taking only 6-8 months to reach market size. In fact, there is at least one major land-based oyster hatchery operating in Hawaii, which benefits from these high growth rates.

Native Hawaiian Aquaculture Site Permitting: Fishponds built by the original inhabitants of Hawaii were among the most sophisticated examples of aquaculture used by pre-historic native Pacific peoples. Rock walls were built along the shoreline to form ponds. The walls were made of porous lava rock, and allowed for saltwater from the ocean to enter and exit the ponds, cleaning them through tidal flushing while preventing fish from escaping. The most widely cultivated species were flathead mullet (*Mugil cephalus*), milkfish (*Chanos chanos*) and moi (*Polydactylus sexfilis*). There were once hundreds of fishponds in use across the Hawaiian Islands, but by the turn of the 20th century only about a hundred remained. Today, most fishponds are in disrepair.

The State of Hawaii, the NOAA Pacific Regional aquaculture coordinator, and various federal agencies and the environmental non-governmental organization Conservation International are collaborating on a general permit to make it possible to restore Hawaiian fishponds for cultural heritage, subsistence, and commercial production. The next step is to develop a general permit from the ACOE to allow pond restoration.

Part of the goal of this project is to introduce a broad segment of the population to aquaculture, through cultural and outreach activities, and foster grass-roots familiarity and support for aquaculture in Hawaii, a state with great marine aquaculture potential.

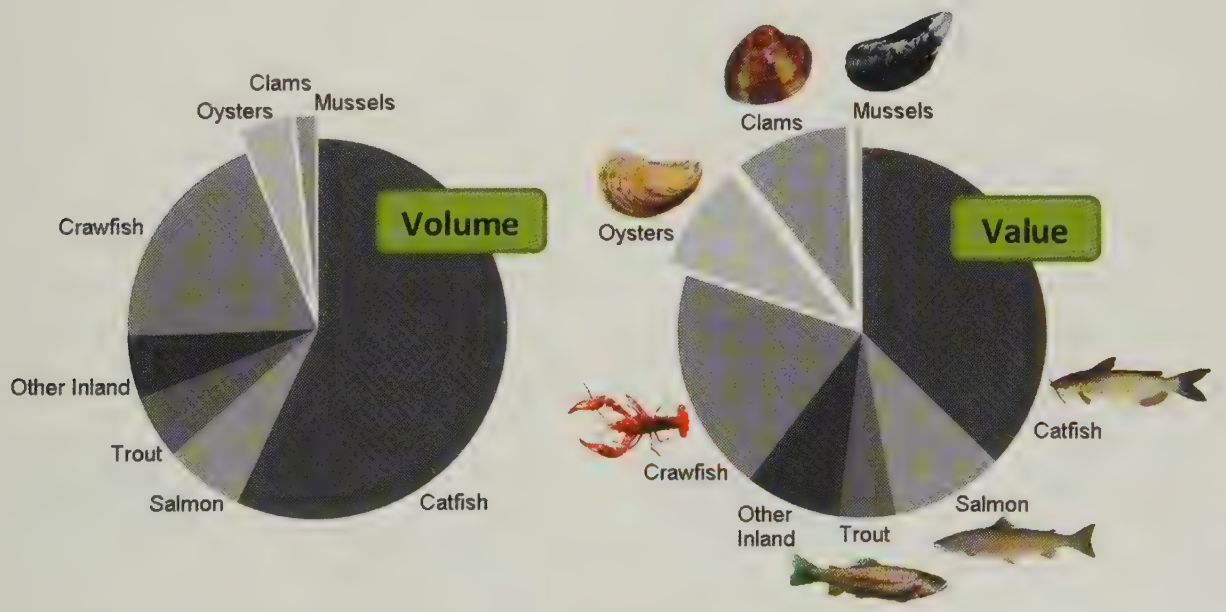


Fig. 1.

Production Trends

The main species for aquaculture production in the U.S. are trout, tilapia, catfish, crawfish, shrimp, salmon, oysters, mussels, and clams. The value of U.S. aquaculture showed 3% average year-over-year growth over the last 15 years, though total volume fell by an average of 0.5%. The last year data are available is for 2011. From 2010 to 2011, total U.S. aquaculture went down -18.8% by volume (to 277,335 metric tons) but grew 4.2% by value (to 1.34billion dollars).

By volume, most of U.S. aquaculture is from land-based ponds growing catfish, crawfish, and trout. Marine species make up a very small proportion of aquaculture production by volume. However, marine species make up a disproportionately large part of the value of aquaculture in the U.S. Shellfish in particular are produced in relatively smaller quantities but are high-value products.

Mariculture[*1]: Major U.S. marine species are Atlantic salmon (*Salmo salar*), mussels (mainly blue mussels, *Mytilus edulis*), clams (largely hard clams *Mercinaria mercinaria* and Manila clam *Venerupis philippinarum*, and geoduck *Panopea generosa*), and oysters (mainly *Crassostrea gigas* and *Crassotrea virginica*). Saltwater shrimp are excluded from mariculture because they are raised in inshore ponds.

From 2010 to 2011 mariculturewent down -12.4% by volume (to 35,739 metric tons) while growing 0.3% by value (to 314 million dollars). However, from 2009-2010, mariculture grew 21.6% by volume (to 40,823 metric tons) and 28.5% by value (to 312 million dollars). The 15-year average growth for mariculture is 4% by volume and 5% by value.

1. Finfish

Atlantic salmon mariculture in the U.S. takes place in Maine and Washington State. Salmon from hatcheries are raised in tanks until they reach 40-120 grams, and then are transferred to marine net-pens for grow-out. From 2010-2011 salmon production decreased -4.8% to 18,595 metric tons, and grew 5.1% by value to 104 million dollars. The average year-over-year growth over the last 15 years is 6% by volume and 11% by value.

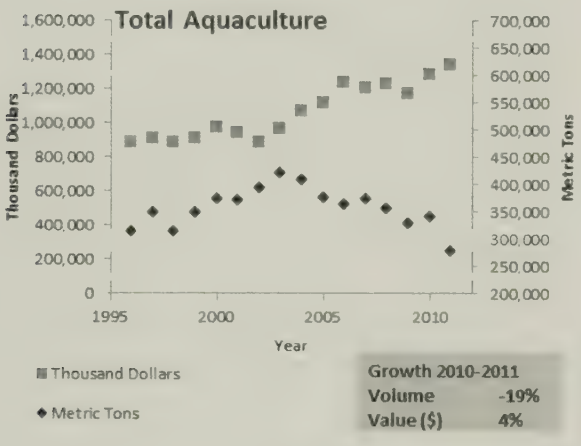


Fig. 2.

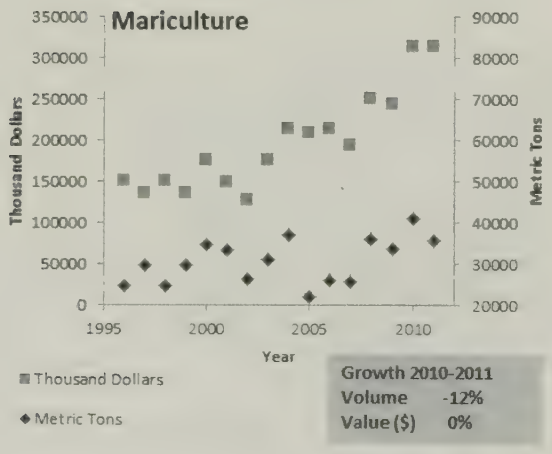


Fig. 3.

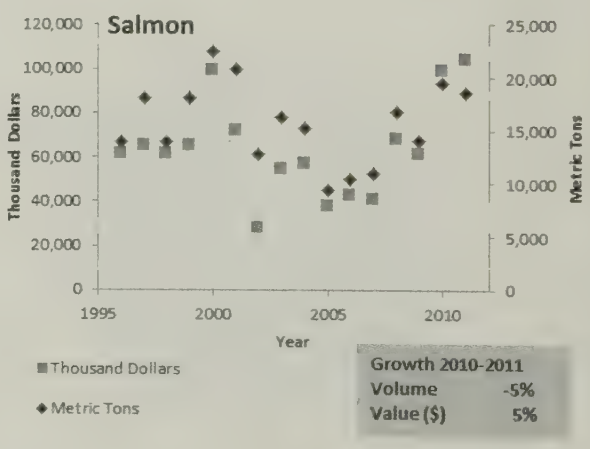


Fig. 4.

[*1] We define Mariculture in this section as species cultured in the marine environment (e.g. oceans, bays, salt-water estuaries).

Other marine finfish species are cultured, but due to issues such as the small number of farms, consistent data are not available. To protect the privacy of businesses, the USDA ARS cannot report detailed statistics on farms when there are so few of them that it would be possible to identify individual farms from the data. White sturgeon are cultured (*Acipenser transmontanus*). Species in the early stages of commercializationor in research and development include sixfinger threadfin (*Polydactylus sexfilis*), commonly known in Hawaii as moi, cobia (*Rachycentron canadum*), yellowtail amberjack (*Seriola rivoliana*), Atlantic cod (*Gadus morhua*), yellowtail amberjack (*Seriola lalandi*), red drum (*Sciaenops ocellatus*), sablefish (*Anoplopoma fimbria*), California flounder (*Paralichthys californicus*), summer flounder (*Paralichthys dentatus*), yellowfin tuna (*Thunnusalbacares*) and Florida pompano (*Trachinotus carolinus*).

2. Shellfish

Clam culture in the U.S. is largely hard clam (*Mercinaria mercinaria*) and Manila clam (*Venerupis philippinarum*), with some native geoduck clams (*Panopea generosa*) grown in the Pacific Northwest. Hard clam culture in the U.S. involves seed production, nursery stage, and final grow-out. Producers rely on seed from hatcheries. At the nursery stage, juvenile clams have a protected environment where they can grow to a size that provides optimum success for survival once placed into a natural setting for grow-out. Once juvenile clams are 7–15 mm, they are placed into the final grow-out system until they reach a harvest size of 50 mm. For grow-out, clams are placed into different structures (e.g. trays, pens, bags) and secured to the substrate of intertidal or subtidal areas. Harvesting occurs by either collecting trays/bags or by raking the substrate.

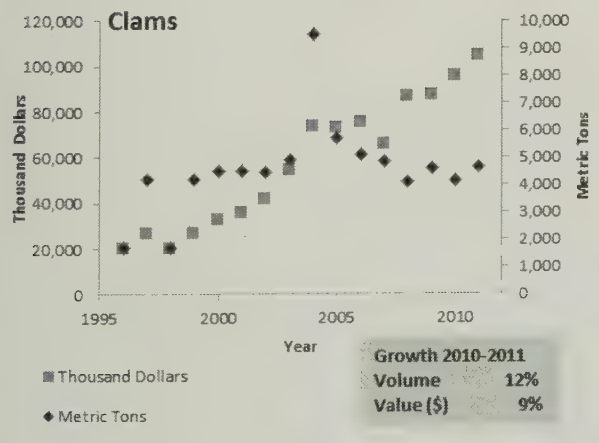


Fig. 5.

Geoduck seed are produced in hatcheries, and juveniles are raised in the hatchery until they reach about 5–12mm in size. Geoduck are next raised in a nursery where they grow large enough for survival outdoors. Geoduck seed are planted on intertidal beach in PVC pipes. The pipe is placed in the sediment, about 30cm deep, with 10 cm left at the surface. Juvenile geoducks are very susceptible to predators and the pipe protects them until they are large enough to evade predators on their own. On many farms, a net is placed over all of the tubes to provide further protection from predators. Two to three juveniles are placed into each pipe. While protected, the geoduck will grow and burrow itself into the sediment. After 1–2 years, the netting is removed since the geoducks are large and deep enough to be safe from predators. The grow-out stage can take 4 to 7 years for a geoduck to reach harvest size of 2 pounds.

From 2010–2011 clam production increased 12% to 4,683 metric tons, and grew 9.3% by value to 104 million dollars.The average year-over-year growth over the last 15 years is 18% by volume and 13% by value.

Oyster culture in the U.S. mainly consists of two species. The Pacific oyster *(Crassostrea gigas)* is the predominant oyster grown on the west coast, and the Virginia oyster (*Crassotrea virginica*) is the most commonly raised oyster on the east coast. However, on both coasts other species are raised in smaller numbers, such as the native Olympia oyster (*Ostreola conchaphila*) on the west coast. The majority of oyster farms rely on hatchery production of seed, though wild seed collection also occurs. Oysters are grown by on-bottom, off-bottom, or suspended-culture techniques. On-bottom culture is used when a site has a suitably firm substrate in an intertidal or sub-tidal area. Off-bottom culture requires seed to be put into mesh bags or trays and attached to rope and wood frames in the intertidal zone to be

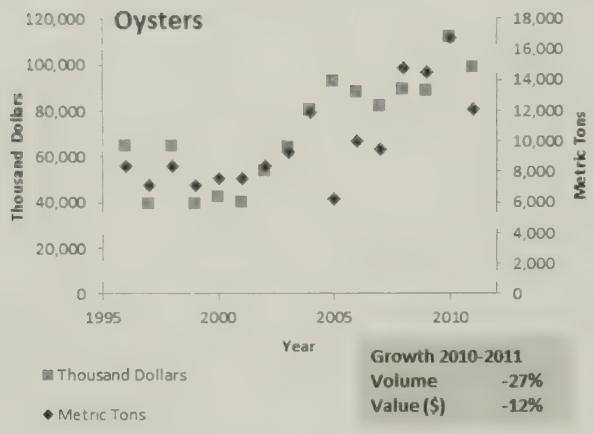


Fig. 6.

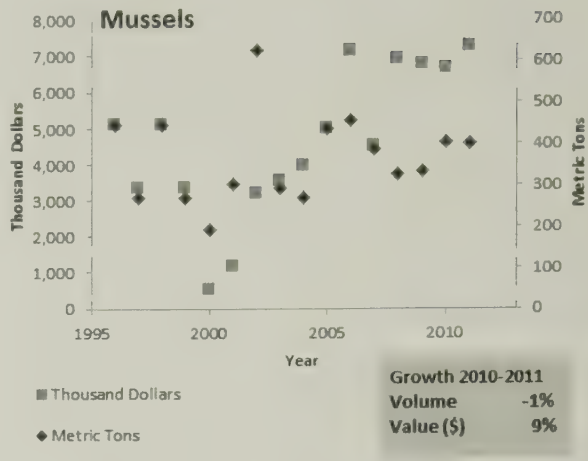


Fig. 7.

grown to market size. Suspended culture is where oyster seed is hung from longlines and attached to horizontal lines or rafts in deeper waters. Harvest typically occurs at a size of around 75mm.

From 2010-2011 oyster harvest dropped -27.8% to 12,062 metric tons, and -11.9% by value to 98.4 million dollars. However, in the previous year, compared to 2009, 2010 saw 15.0% growth by volume to 16,721 metric tons, and 26.4% growth by value to 111.7 million dollars. The average year-over-year growth over the last 15 years is 6% by volume and 6% by value.

Mussels are farmed either on- or off-bottom. On-bottom mussel farming involves seeding sea bottom areas, and dredging mussels for harvest. There are several methods of off-bottom farming, including longline culture and raft culture. From 2010-2011 mussel production decreased -0.7% to 402 metric tons, and grew 9.3% by value to 7.2 million dollars. The average year-over-year growth over the last 15 years is 8% by volume and 20% by value.

Inland Aquaculture[*2]: Major U.S. inland finfish aquaculture species are channel catfish (*Ictalurus punctatus*), Rainbow trout (*Oncorhynchus mykiss*), hybrid striped bass (*Morone saxatilis x M. chrysops*), and tilapia (*Tilapia spp.*). Farmed shellfish include the red swamp crawfish (*Procambarus clarkii*) and whiteleg shrimp (*Litopenaeus vannamei*).

From 2010 to 2011 inland aquaculture went down

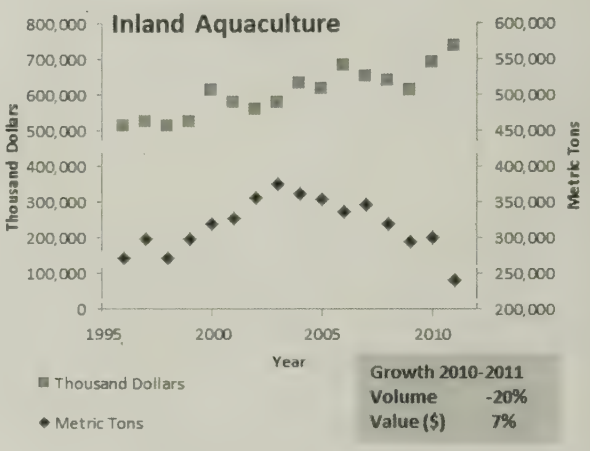


Fig. 8.

19.7% by volume (to 241,596 metric tons) while growing 7.2% by value (to 737 million dollars). The average year-over-year growth over the last 15 years is -0.3% by volume and 3% by value.

1. Finfish

Channel catfish (*Ictalurus punctatus*) are the predominant aquacultured species in the U.S., and only Channel catfish can be sold as 'catfish' in U.S. markets. Catfish are grown primarily in embankment or levee ponds, where the earth that is removed to dig the ponds is used to build levees or embankments to encircle the ponds. Ponds range in size from 0.04 km^2 and 2m deep to 0.1 km^2 and 1m deep.

*2. In this section, inland aquaculture refers to the culture of freshwater species as well as marine species grown in land-based systems.

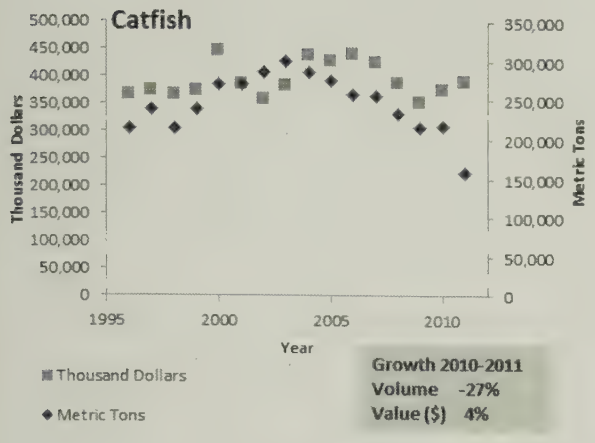


Fig. 9.

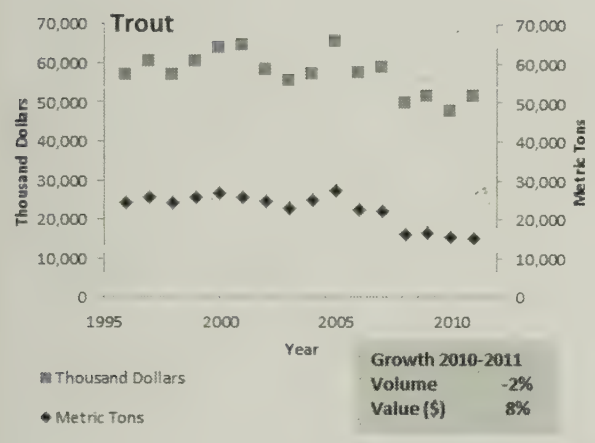


Fig. 10.

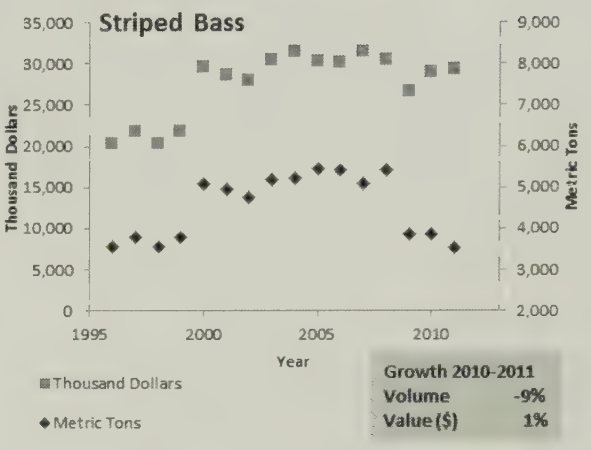


Fig. 11.

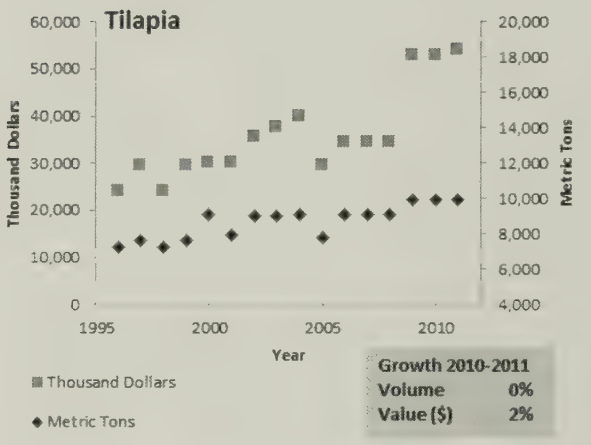


Fig. 12.

From 2010 to 2011 catfish production went down-27.3% by volume (to 157,942 metric tons) while growing 4.2 % by value (to 391 million dollars). The average year-over-year growth over the last 15 years is -1% by volume and 1% by value.

Rainbow trout (*Oncorhynchus mykiss*) are predominantly raised in raceways, though some farms use ponds or recirculating systems. Raceways, also known as flow-through systems, are essentially artificial streams. Gravity flows water from natural streams through the concrete raceways, and the water is often filtered to remove waste products before being released back into the natural stream. Steelhead trout (*Oncorhynchus mykiss*) are sea-run rainbow trout, and can be raised in net-pens in rivers and the marine environment. From 2010 to 2011 trout production went down -1.8% by volume (to 15,112 metric tons) while increasing 7.9% by value (to 51 million dollars). The average year-over-year growth over the last 15 years is -3% by volume and 0% by value.

Hybrid striped bass are produced in the hatchery by fertilizing female white bass (*Morone chrysops*) eggs with sperm from male striped bass (*Morone saxatilis*). Hybrid striped bass have greater tolerance to extremes in temperature and dissolved oxygen than either of its parents and is thus better suited for pond culture.The majority of hybrid striped bass producers in the southern region raise their fish in freshwater ponds, but cages and tanks are also used for hybrid striped bass production.

From 2010 to 2011 hybrid striped bass production decreased 9.1% by volume (to 3,516 metric tons) while growing 1.4% by value (to 28 million dollars). The average year-over-year growth over the last 15 years is 1% by volume and 3% by value.

Tilapia are grown in ponds or tanks in the U.S., and production did not change between 2010 and 2011, though there was an increase in value of 1.7%. In 2011, 9,979 metric tons of tilapia worth 54 million dollars were produced. The average year-over-year growth over the last 15 years is 2% by volume and 7% by value.

2. Shellfish

Crawfish are the predominant cultured crustacean in the U.S., and are cultivated and consumed for food in several southern states. Louisiana dominates the aquaculture and wild harvest crawfish industry. Crawfish are grown in shallow ponds 20 to 60 cm deep, and ponds are flooded and drained each year. Crawfish in the U.S. are not fed formulated feeds, and instead primarily consume plant material as forage. In order to provide crawfish with plant material to feed upon, they are often double-cropped or rotated with field crops, most commonly rice but also soybeans and sorghum. From 2010 to 2011 crawfish production grew 0.9% by volume (to 53,435 metric tons) while growing 15.9% by value (to 206 million dollars). The average year-over-year growth over the last 15 years is 13% by volume and 17% by value.

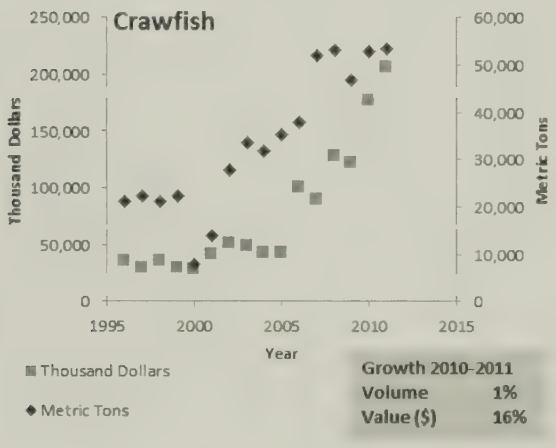


Fig. 13.

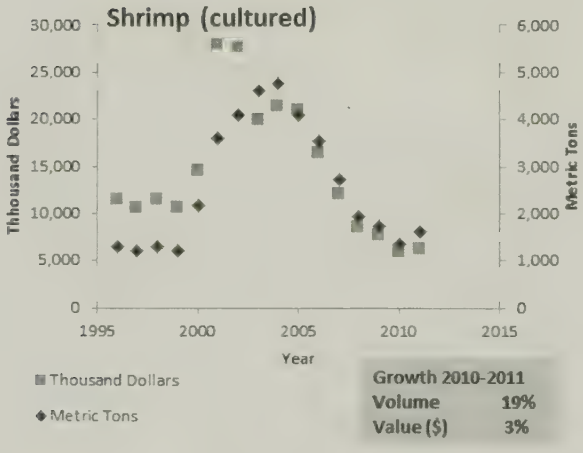


Fig. 14.

Shrimp in the U.S. are mostly wild-caught. However, both marine (penaeidae) and freshwater shrimp species are grown in ponds, tanks, and recirculating systems. Whiteleg shrimp (*Litopenaeus vannamei*) are the most commonly cultured shrimp species, though culture of the Malaysian prawn (*Macrobrachium rosenbergii*) in land-based systems is increasingly popular. From 2010 to 2011 shrimp aquaculture grew 19% by volume (to 1,612 metric tons) while growing 3.2% by value (to 6.1 million dollars). The average year-over-year growth over the last 15 years is 5% by volume and -0.6% by value.

3. Other land-based culture of marine species

There are several ongoing experimental and commercial land-based marine finfish culture operations in the U.S.: notably barramundi (*Lates calcarifer*) has been commercialized in a large land-based facility in Massachusetts. Several non-native species of sturgeon (*Acipenser baeri, A. gueldenstaedtii*) have been cultured in commercial and demonstration projects in Florida (e.g. Mote Marine Laboratory). Additionally, work to commercialize cobia (*Rachycentron canadum*) and black sea bass (*Centropristis striata*) continues in land based recirculating systems in Virginia.

Annotated Bibliography

Lowther A. (editor), 2013: Fisheries of The United States 2012. Current Fishery Statistics No. 2012. National Marine Fisheries Service Office of Science and Technology. http://www.st.nmfs.noaa.gov/Assets/commercial/fus/fus12/FUS2012.pdf

The annual Fisheries of the United States is the official published source of fisheries statistics for the US. The 2012 report gives preliminary 2012 data on US commercial fisheries, and the final report for

2012 on US recreational fisheries. US aquaculture statistics are reported for 2011. Summary statistics are also provided on US production of processed fishery products, domestic supply and per capita consumption, foreign trade, and world fisheries production.

Kapetsky J. M., Aguilar Manjarrez J., and Jenness J., 2013: A global assessment of potential for offshore mariculture development from a spatial perspective. FAO Fisheries and Aquaculture Technical Paper No. 549. Rome, FAO. 181 pp

The FAO produced this document to highlight that mariculture, specifically offshore aquaculture, offers significant opportunities for sustainable food production but is underutilized worldwide. The report states that offshore aquaculture could foster development of many coastal communities, especially in regions with limited freshwater and arable land. This report measures and compares for all maritime nations the current status and potential for offshore mariculture development from a spatial perspective, and identifies nations with high but unrealized offshore potential.

水研センター研報，第40号，33－38，平成27年
Bull. Fish. Res. Agen. No. 40, 33－38, 2015

The Challenge of Reconstructing Coho Salmon Aquaculture after the Great East Japan Earthquake and Tsunami in 2011

Ikutaro SHIMIZU[*1], Tsuyoshi TANAKA[*2], Hideki MIURA[*3], and Kohichi SAOTOME[*4]

Abstract: The wholesale price of farmed salmon in local fish markets showed higher prices before the tsunami in 2011. Though farmed coho salmon started being landed again by the support of national funding in 2012, the price of the farmed salmon slumped. A higher price is necessary to maintain the farming facilities for management. This paper aims to clarify the causes of the price drop of Sanriku coho salmon and the issues in the reconstruction process of coho salmon aquaculture. The most important cause of the price drop of Sanriku coho salmon was that consumers' purchasing patterns changed from Sanriku coho salmon to imported salmon due to the lack of Sanriku coho salmon in 2011. We hope to supply fresh Sanriku coho salmon at prices between imported fresh Atlantic salmon and frozen rainbow trout in consumer markets. It is necessary to improve the traditional aquaculture system and to develop brand value for Sanriku coho salmon. Sales promotion is expected to drive the price of the fresh coho salmon up to a fair level.

Key words: coho salmon, aquaculture, Miyagi prefecture, tsunami, reconstructing, and price analysis

Aquaculture of coho salmon (*Oncorhynchus Kisutsh*) is one of the most important fisheries in Miyagi prefecture, Tohoku, where the annual production was more than 10,000 tons until 2010 (Fig. 1). However, the Great East Japan Earthquake hit the Pacific coast area of eastern Japan on March 11 in 2011. The tsunami destroyed nearly all the farming facilities, local fish markets, and processing firms in the Sanriku region, and suspended coho salmon farming. One year later, farmed coho salmon started being landed again with help from national funding. Fortunately, the tsunami did not affect the coho salmon juveniles which were located inland. While the wholesale prices of the farmed salmon in local fish markets showed higher prices (more than 400 yen/kg) before the tsunami, the price of the farmed salmon slumped (less than 200 yen/kg) in 2012 (Fig. 2). Prices higher than 370 - 380 yen/kg is necessary to maintain the farming facilities and bread even. Understanding the causes of the slump is necessary to reconstruct Sanriku salmon aquaculture.

This paper clarifies the causes of the price drop of Sanriku coho salmon and the issues in the reconstruction process of coho salmon aquaculture. We conducted interviews at fisheries cooperatives, farming facilities, local fish markets, seafood processing firms, and wholesale markets in Miyagi prefecture in September and October 2012, and general merchandising stores in the Tokyo area in November 2012. In addition, the relational expressions of salmon price and fishmeal price were

2015年1月30日受理 (Received on January 30, 2015)
*1 National Research Institute of Fisheries Science, Fisheries Research Agency, 2-12-4 Fukuura, Kanazawa, Yokohama, Kanagawa, 236-8648 Japan (Current affiliation: Hokkaido National Fisheries Research Institute, Fisheries Research Agency, 2-4-1 Nakanoshima 2jo, Toyohira, Sapporo, Hokkaido, 062-0922 Japan)
E-mail: ikutaro@affrc.go.jp
*2 Fishing Industry and Communities Promotion Organization, 1-27 Kaisei, Ishinomaki, Miyagi, 986-0032 Japan
*3 Japan Fisheries Cooperatives, 1-1-12 Uchikanda, Chiyoda, Tokyo, 101-8503 Japan
*4 Tohoku National Fisheries Research Institute, Fisheries Research Agency, 3-27-5 Shinhamacho, Shiogama, Miyagi, 985-0001 Japan (Current affiliation: Fisheries Agency, Ministry of Agriculture, Forestry and Fisheries, 1-2-1 Kasumigaseki, Chiyoda, Tokyo, 100-8950 Japan)

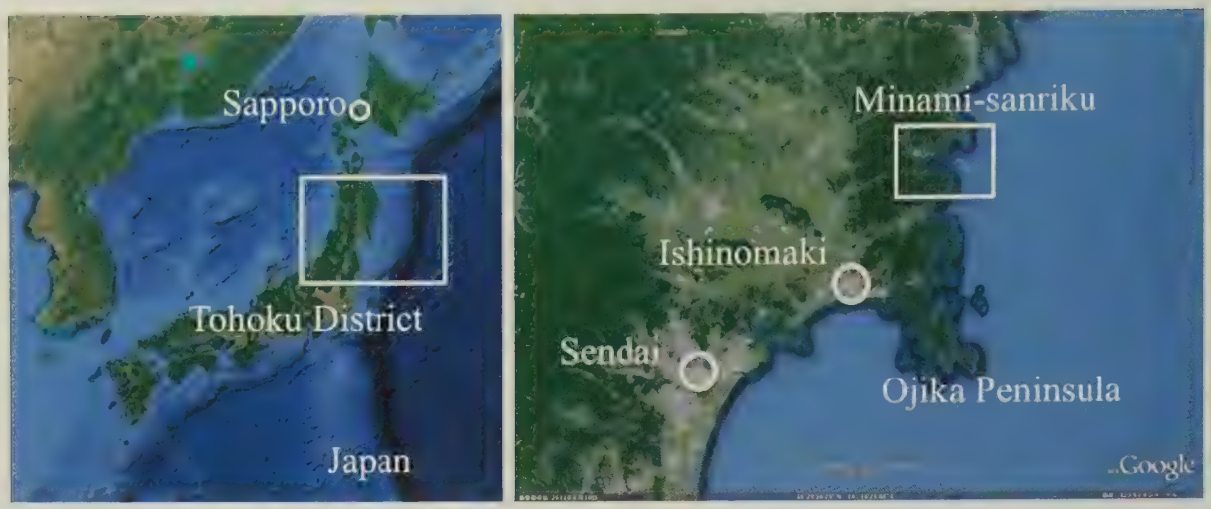


Fig. 1. Maps of Japan on the left and the Sanriku region in the Tohoku district on the right prepared by the Google Maps.

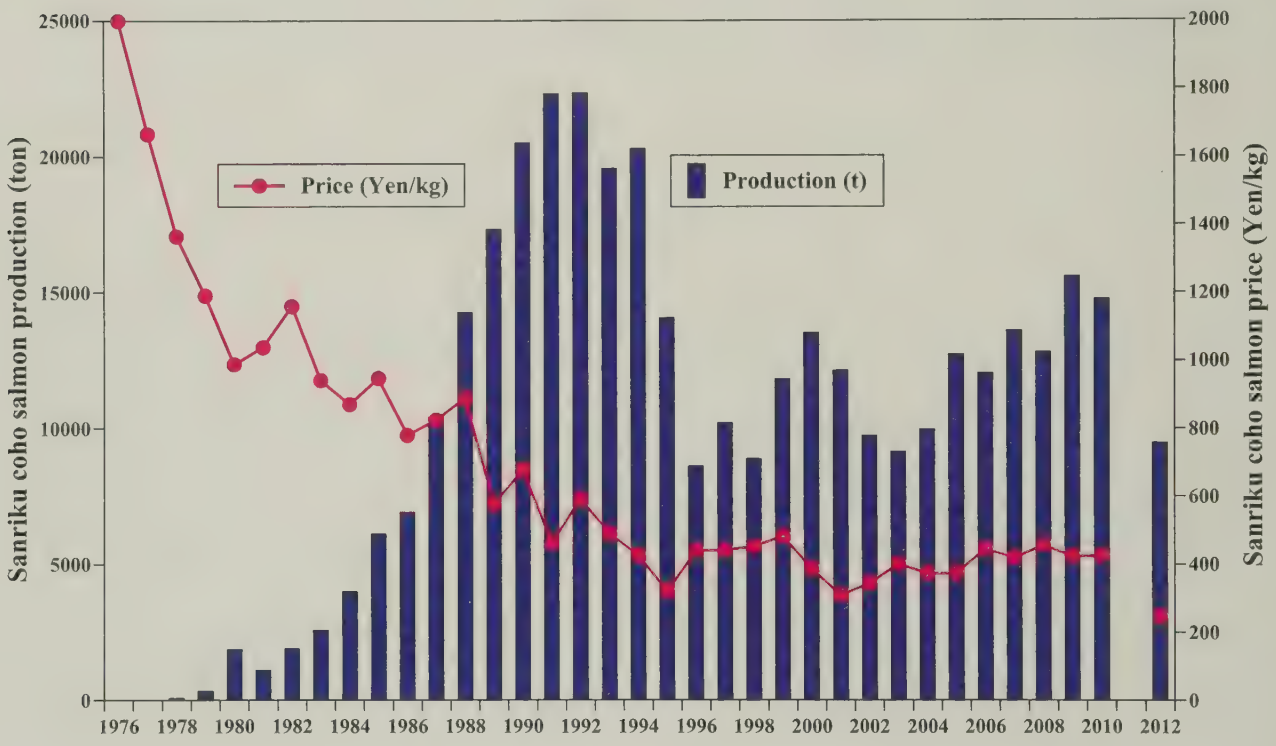


Fig. 2. Variation of production and price of Sanriku farmed coho salmon from 1976 to 2012. The landing of 2011 was nothing by the damage of tsunami. These data were supplied by JF-Miyagi, 2012: Data of landing amounts and values of farmed coho salmon in Miyagi prefecture.

measured with the software R (http://www.r-project.org).

Process of coho salmon aquaculture in Japan

Domestic eyed eggs of coho salmon are supplied from inland hatcheries (Koshimizu and Sarabetsu) in Hokkaido and a part of eyed eggs are imported from a company in the US (AquaSeed Corporation in Washington) to inland fish farms in the Tohoku district. Coho salmon fry are produced by inland fish farms in Iwate and Miyagi prefectures from December to the next October (Fig. 3). Coho salmon fry (total length: 140 -180 cm) are

Inland farming
Egg collection (November) in Hokkaido
Eyed egg transportation (December) to Miyagi and Iwate
Hatch out (10-15 days later)
Fry farming (140-180 g until late October, next year)
Marine farming
Seawater adaptation
Feeding management (November to next July)
Landing (bigger than 1.5 kg BW from April to July)
Local fish markets or Processing firms in Sanriku area
Consumer markets in Japan

Fig. 3. Farming schedule of Sanriku coho salmon. Inland farming is consisted from eyed egg production in Hokkaido and fry farming in Miyagi and Iwate prefectures. Marine farming is operated along Sanriku coast in Miyagi prefecture.

transported to marine cages in the Sanriku region. The fish farmers begin to land coho salmon after they grow bigger than 1.5 kg body weight from April until early August. Landed farmed coho salmon are transported to processing facilities and they are supplied to consumer markets around Japan.

Causes of the price drop of Sanriku coho salmon

Based on the interviews, the causes of the price drop in domestic farmed coho salmon (Sanriku coho salmon) can be as follows;

(1) The mass imports of Chilean farmed coho salmon starting autumn 2011 reduced the domestic wholesale price,
(2) Low seawater temperature in the winter 2012 suppressed the growth of Sanriku coho salmon,
(3) Late landing of Sanriku coho salmon in the spring 2012 affected supply in June,
(4) The spread of rumors about radiation caused by the accident at Fukushima nuclear power plant decreased the demand for Sanriku coho salmon,
(5) Avoiding prolonged cold storage and increase in fresh supply of Sanriku coho salmon reduced the price of the farmed coho salmon, and
(6) The discontinuation of the supply of Sanriku coho salmon in 2011 changed consumers' purchasing pattern from Sanriku coho salmon to imported salmon.

Issues about coho salmon aquaculture

The price of Sanriku coho salmon is synchronized with the import price of frozen farmed coho salmon and frozen farmed rainbow trout from Chile, and has decreased since the 1980s (Fig. 4). The import price of frozen Chilean coho salmon (IPCCS) is synchronized with the import price of frozen Chilean rainbow trout (IPCRT). IPCCS (yen/kg/year) was explained by the imports (tons/year) of frozen Chilean coho salmon (IQCCS) and IPCRT (yen/kg/year) from 1989 to 2010. The data was used by the

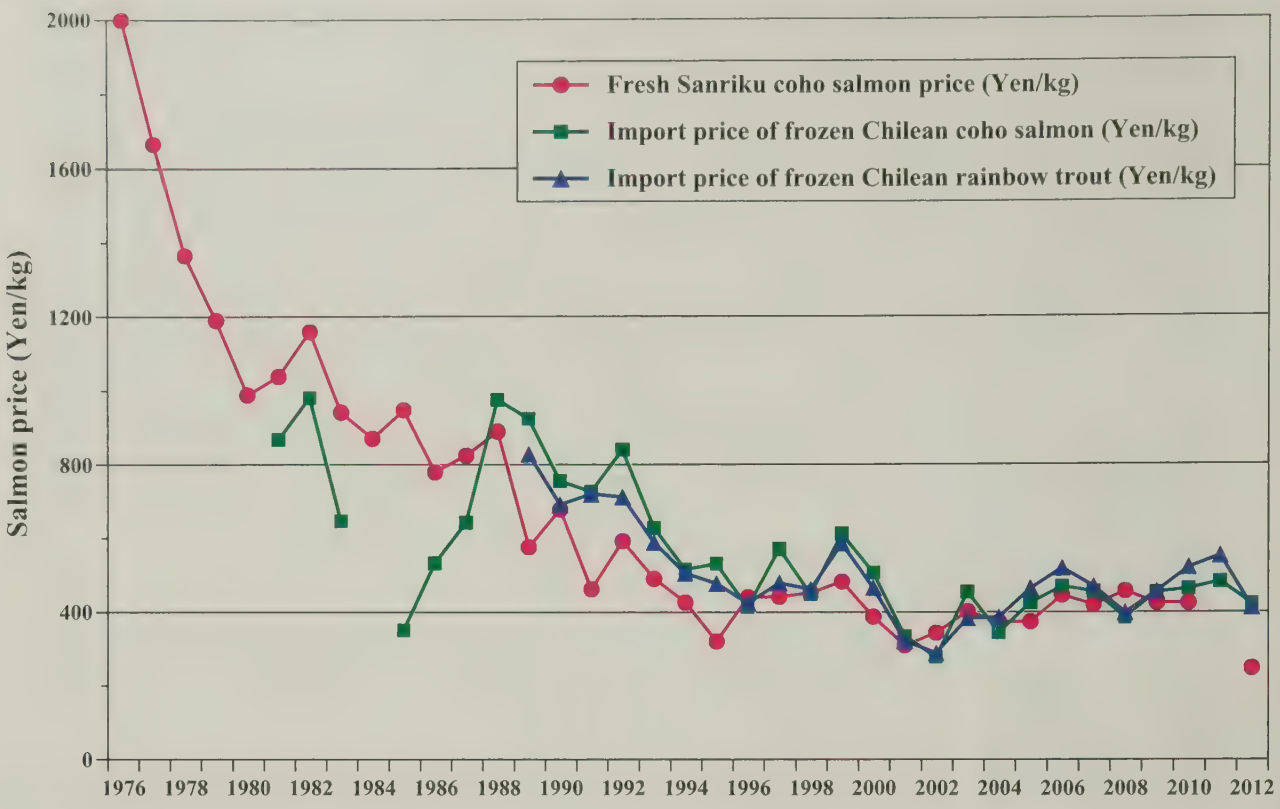

Fresh Sanriku coho salmon price (Yen/kg)
Import price of frozen Chilean coho salmon (Yen/kg)
Import price of frozen Chilean rainbow trout (Yen/kg)
Salmon price (Yen/kg)
2000
1600
1200
800
400
0
1976 1978 1980 1982 1984 1986 1988 1990 1992 1994 1996 1998 2000 2002 2004 2006 2008 2010 2012

Fig. 4. Prices of fresh Sanriku coho salmon, frozen imports of coho salmon and rainbow trout from Chile between 1976 and 2012. The prices of fresh Sanriku coho salmon were supplied from JF-Miyagi and import prices were provided by the trade statistics, Ministry of Finance of Japan.

trade statistics, Ministry of Finance of Japan Trade statistics (http://www.customs.go.jp/toukei/).

This study employed a conventional double-log demand equation model with the estimation results as follows:

$$\text{Log (IPCCS)} = 1.395 - 0.077\text{Log (IQCCS)} + 0.910\text{Log (IPCRT)}$$
$$(1.308)\ (-2.104)^{*}\qquad (7.752)^{***}$$

Adjusted $R^2 = 0.928$, DW = 2.54, n = 22

The statistical significant code: ***, **, and * suggests p-value under 0.005, under 0.01, and under 0.1, respectively. DW is Durbin-Watson ratio.

The price (yen/kg/year) of fresh Sanriku coho salmon (PSCS) was explained by the import price (yen/kg/year) of frozen Chilean rainbow trout (IPCRT) from 1989 to 2010. The data was used by the local market price of JF (Japan Fisheries Cooperatives) −Miyagi and the trade statistics (http://www.customs.go.jp/toukei/), Ministry of Finance of Japan.

This regression model was estimated by step AIC as follows:

$$\text{Log (PSCS)} = 2.494 + 0.578\text{Log (IPCRT)}$$
$$(4.114)^{***}(5.911)^{***}$$

Adjusted $R^2 = 0.618$, n = 22

The result shows the import price of frozen Chilean coho salmon increases by 0.91% as the import price of frozen Chilean rainbow trout increases 1%. In addition, the price of fresh Sanriku coho salmon increases by 0.58% as the import price of frozen Chilean rainbow trout increases 1%.

In the Japanese salmon market, the highest priced salmon is fresh Atlantic salmon imported from Norway (Fig. 5). The second highest priced salmon are frozen sockeye salmon imported from Alaska, frozen rainbow trout or frozen coho salmon imported from Chile. The lowest priced salmon are domestic chum salmon and export chum salmon to China. One of the most important characteristics of Sanriku coho salmon aquaculture is market differentiation by being a fresh product. In addition, the supply of fresh seafood in spring has been less than other seasons in the Sanriku region. We hope to move the

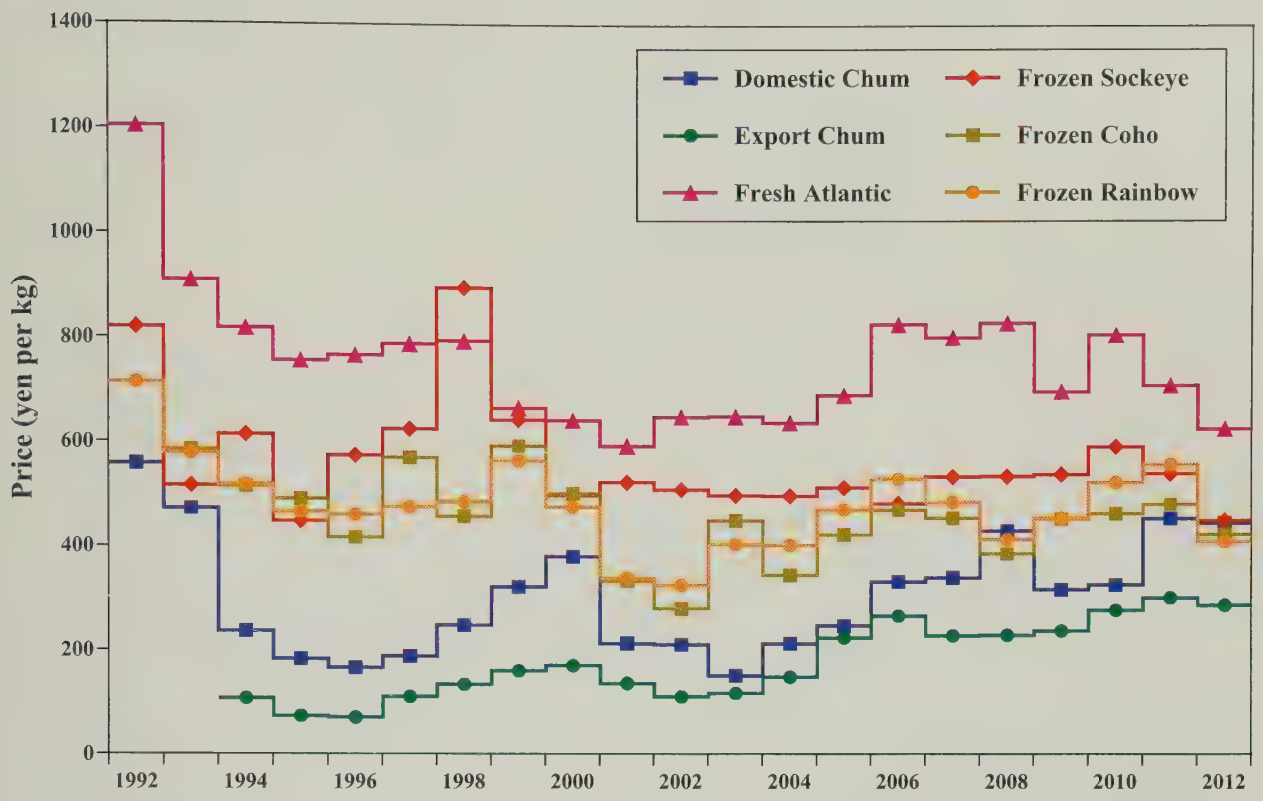


Fig. 5. Prices of salmons in Japanese market. The highest price is fresh Atlantic salmon from Norway. The secondary prices are frozen sockeye salmon imported from Alaska, and frozen rainbow trout and frozen coho salmon imported from Chile. The third prices are domestic chum salmon in Japan and export chum salmon to China. The prices were provided by the annual report (Hokkaido Stationary Net Fisheries Association, 2012: Annual report of salmon distribution in Hokkaido. 96 pp.).

price of fresh Sanriku coho salmon to be between fresh Atlantic salmon and frozen rainbow trout in consumer markets.

Issue about fishmeal in Japan

One of the common issues about Sanriku coho salmon aquaculture system (from eyed eggs production, and fry production, to seawater farming) is the impact on feed costs due to import price of foreign produced fishmeal. The price increase of imported fishmeal has impacted the economics of inland and seawater fed aquaculture. The import price (yen/kg/year) of fishmeal in Japan (JFIP) was explained by the export quantity (tons/year) of Peruvian fishmeal (PFEQ), the fishery production (tons/year) of Peruvian anchovy (PAFQ) and the import quantity of fishmeal in China (CFIQ). The data was used from 1984 to 2009 of the FAO statistics, FishStatJ[*5]. This model with estimation results by step AIC is as follows:

$$\begin{aligned}\text{Log (JFIP)} &= 8.860 - 0.071\text{Log (PAFQ)} \\ &\quad (7.336)^{***}\ (-1.465) \\ &\quad -0.410\text{Log (PFEQ)} + 0.182\text{Log (CFIQ)} \\ &\quad (-3.226)^{**}\qquad (3.427)^{**}\end{aligned}$$

Adjusted $R^2 = 0.635$, $n = 26$

In addition, the import quantity (tons/year) of fishmeal in Japan (JFIQ) was explained by the fish aquaculture production (tons/year) in Japan (JAPQ) and the export quantity (tons/year) of Peruvian fishmeal (PFEQ). The data was used from 1979 to 2009 of the FAO statistics, FishStatJ. This demand model is defined below:

$$\begin{aligned}\text{Log (JFIQ)} &= 27.001 + 2.389\text{Log (JAPQ)} \\ &\quad (-2.057)^{*}\ (2.089)^{*}\end{aligned}$$

[*5] (http://www.fao.org/fishery/statistics/software/fishstatj/en), Software for fishery statistical time series of FAO

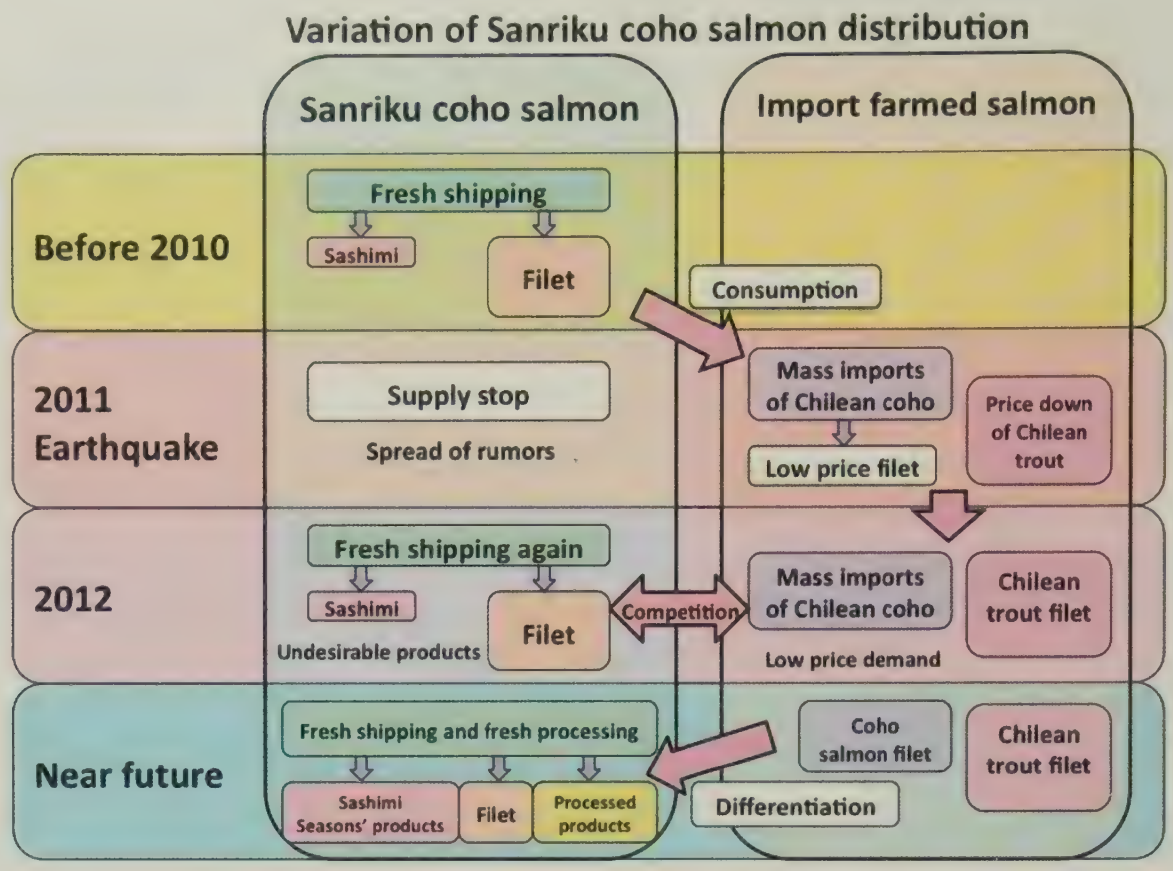


Fig. 6. Distribution image of Sanriku farmed coho salmon and import farmed salmon from Chile in Japanese market before and after the earthquake of 2011.

$$+0.663\mathrm{Log}\,(\mathrm{PFEQ})$$
$$(3.930)^{***}$$

Adjusted $R^2 = 0.614$, DW = 1.86, n = 31

The import price of fishmeal in Japan decreases by 0.41% as the export quantity of Peruvian fishmeal increases 1%. The import quantity of fishmeal in Japan increases by 2.4% as the fish aquaculture production in Japan increases 1%. We hope fishmeal production would be stable in the near future. At the same time, the development of feed with low fishmeal rate will be expected.

Conclusions

It is important to supply fresh salmon for consumer markets by the Golden Week holidays (from late April to early May) in Japan. It is necessary to improve the traditional aquaculture system and to create a new brand value for Sanriku coho salmon. Sales promotion is expected to drive the price of fresh coho salmon up to a fair level. We believe that the development of Sanriku coho salmon aquaculture contributes to the reconstruction of regional industries in Miyagi prefecture. All of the Chilean farmed coho salmon and rainbow trout are imported frozen. We believe that one of the most important characteristics of Sanriku coho salmon for market differentiation is freshness (Fig. 6).

水研センター研報，第40号，39-49，平成27年
Bull. Fish. Res. Agen. No. 40, 39-49, 2015

Development of a New Type of Fish Diet, Non-Fish Meal Extruded-Pellet

Noriko ISHIDA[*1], Tomohiko KOSHIISHI[*2], Tatsuo TSUZAKI[*3], Koji MAENO[*4], Satoshi KATAYAMA[*5], Minoru SATOH[*5], and Shuichi SATOH[*6]

Abstract: We developed a non-fish meal diet using plant and/or animal protein materials. Three kinds of non-fish meal diets and a control diet containing 50% fish meal were processed. In the non-fish meal diets, the fish meal was replaced with commercially available plant or animal materials and supplemented with taurine and materials for maintaining palatability. These diets were fed to one year old yellowtail (BW 753±96g) in net cages. There was no difference in growth, daily weight gain, daily feeding rate, feed conversion ratio and protein efficiency ratio among the diets. Non-fish meal diets were processed in a factory and dietary properties were studied such as uptake, stomach evacuation rate, and comparative disease resistance in fish fed the experimental diets. In addition, palatability of each substitute protein material for fish was examined and materials to enhance palatability of the non-fish meal diets were clarified. Non-fish meal diets have the potential to support the growth of one year old yellowtail.

Key word: Fish diet, Non-fish meal, Extruded-pellet, Yellowtail, Palatability, Substitute protein material

In Japan, fish meal is the main material in fish diets used in aquaculture and is contained at a level of over 50%. Over 90% of the fish meal used in fish diets for aquaculture is imported. Globally, the price of fish meal has increased dramatically in recent years due to reduced production caused by fishery regulation in South America and the increased worldwide demand for aquacultured fish. The increasing fish meal price threatens of the viability of production for Japanese aquaculture farmers. Therefore, a new type of diet that is independent of fish meal is strongly required. Low or non-fish meal extruded-pellets have been developed. In our preliminary study, we prepared diets containing 10% fish meal, and fed them to cultured yellowtail to compare the fish growth performances to a diet containing 50% fish meal. It was observed that fish growth was similar in both diet groups. Three experimental non-fish meal extruded-pellets were designed and fed to one year old yellowtail which were at an immature and rapidly growing size. We also studied dietary, uptake, stomach evacuate rate, palatability, and comparative disease resistance of fish. Then the meat quality fed the experimental diets was evaluated using a panel sensory test.

Materials and Methods

Experimental diets and processing: The control diet and three kinds of non-fish meal diets were prepared using a variety of ingredients (Table 1) (Tsuzaki *et al.*, 2013 and 2014). The control diet (FM) contained 50% fish meal to provide a suitable balance of amino acids. Fish meal was replaced with commercially available plant (PP) and animal (AP) materials in the non-fish meal diets. Soy protein concentrate, defatted soy bean meal, and corn gluten meal were used for plant materials and pork meal, feather

2015年1月30日受理 (Received on January 30, 2015)
*1 National Research Institute of Fisheries Science, FRA, Yokohama 236-8648, Japan
E-mail: larry@affrc.go.jp
*2 Marubeni Nisshin Feed Co., Ltd., Aichi 441-3605, Japan
*3 National Research Institute of Fisheries and Environment of Inland Sea, FRA, Ehime 794-2305, Japan
*4 Kagoshima Prefectural Fisheries Technology and Development Center, Kagoshima 891-0315, Japan
*5 Tohoku University, Sendai 981-8555, Japan
*6 Tokyo University of Marine Science and Technology, Tokyo 108-8477, Japan

Table 1. Formulation of the experimental diets (%)

Ingredient	Diet group			
	FM	PP-SP	PP-FS	AP-SP
Fish meal [1]	50.0	0	0	0
Soy bean concentrate [2]	0	30.0	30.0	0
Defatted soy bean meal [3]	3.5	11.0	11.0	8.0
Corn gluten meal [4]	0	12.0	12.0	9.0
Krill meal	0	3.0	3.0	3.0
Pork meal [5]	0	0	0	30.0
Feather meal [5]	0	0	0	3.0
Blood meal [6]	0	0	0	3.0
Fish oil [7]	15.4	17.0	17.0	14.0
Wheat	13.0	10.8	10.8	12.0
Defatted rice brain [8]	8.5	0	0	1.8
Tapioca starch [9]	6.6	8.0	8.0	8.0
Vitamin mixture [10]	2.0	2.0	2.0	2.0
Mineral mixture [10]	1.0	1.0	1.0	1.0
Taurine	0	1.0	1.0	1.0
Amino acid mixture [11]	0	2.2	2.2	2.2
Calcium phosphate [12]	0	2.0	2.0	2.0
Enzyme mixture [13] (external addition)	0	(0.2)	(0.2)	(0.2)
Skipjack peptide [14] (external addition)	0	(0.5)	0	(0.5)
Fish sauce [15] (external addition)	0	0	(0.5)	0

[1] Fish meal was made from anchovy meal as the main raw material, imported from Peru.

[2] Soycomil; Archer Daniels Midland Japan Ltd.

[3] Nisshin Oillio Co., Ltd.

[4] Japan Corn Starch Co., Ltd.

[5] Kagoshima Pure Foods Co., Ltd.

[6] APC Japan Co., Ltd.

[7] Tsuji Oil Mills Co., Ltd.

[8] Suzuki Oil Mills Co., Ltd.

[9] Sanguan Wongse Industries Co., Ltd.

[10] See Takagi *et al.* (2005).

[11] See Aoki *et al.* (2000b).

[12] Onoda Chemical Industry Co., Ltd.

[13] Alltech.

[14] Marubeni Nisshin Feed Co., Ltd.

[15] Sakaiovex Co., Ltd.

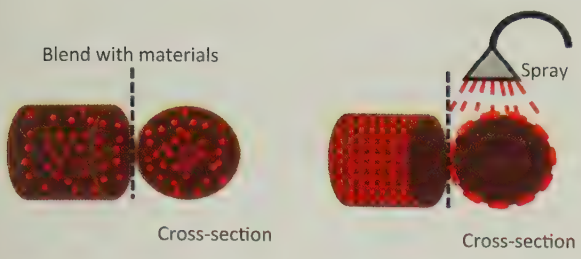


Fig. 1. Method to add palatability materials and enzyme solution to fish pellets. Right is sprayed after being shaped to reduce the material volume. Left is shaped after blending all the materials.

meal, and blood meal were used for animal materials in this study. Taurine was added to the non-fish meal diets at a 1% composition. Experimental diets were carefully shaped using an extruder to have suitable physical properties in the sea and sink at an appropriate rate rather than float.

It is known that yellowtail become less responsive to a diet as the amount of fish meal included is reduced (Aoki *et al.*, 2000a and 2000b). Therefore, two commercially available materials, a skipjack peptide (SP) and a fish sauce (FS) were supplemented to maintain the palatability of the non-fish meal diet. The skipjack peptide is concentrated from the cooking water of skipjack. The fish sauce is made from mackerel gut using a patent-pending method for rapid production. An enzyme solution and amino acid chelated trace metals were added to the non-fish meal diets to improve digestibility and absorption. These palatability materials and enzyme solution were added by a spray method after the extruder processing to minimize the volume (Fig. 1).

Therefore, the three kinds of non-fish meal diets were as follows:

PP-SP was a plant protein based diet with the skipjack peptide as a material to enhance palatability.

PP-FS was a plant protein based diet with the fish sauce as a material to enhance palatability.

AP-SP was an animal protein based diet with the skipjack peptide as a material to enhance palatability.

Two sizes of experimental diets, 13mm and 15mm grain diameter, were prepared at the Kagoshima plant of Marubeni Nisshin Feed Co., Ltd. There were minimal differences in the general composition among the four experimental diets. Small differences were seen in the fatty acid composition. AP-SP had 16% of n-3 PUFA while the other diets ranged from 22–27%. AP-SP also had 41% of total monoenes, higher than the 30–35% found in the other diets (Table 2).

Feeding experiment: One year old yellowtails which originated from seedling production in Goto Island, Nagasaki Prefecture were used in the experiments (Tsuzaki *et al.*, 2013 and 2014). The average body weight of 220 fish was 753 ± 96g. Four groups of 55 fish fed different experimental diets to satiation three times a week for six months from July to January were reared in four 4 m x 4 m replicate net cages on the sea surface. After January fish continued to be fed with the leftover experiment diet until March.

The feeding experiment was divided into two periods, summer season (July to September) and autumn season (October to December). Sampling and analysis were performed for each period.

Body weight and fork length were monthly measured for 20–25 live fish at random from every group. At the end of the experiment, all the fish were sampled and weighed and the following data were measured or calculated.

Condition factor = $(W / FL^3) \times 10^3$

Weight gain ratio (%) = $(TWt + SDW - TW0)/TW0 \times 100$

Daily weight gain ratio (%/day) = $(TWt + SDW - TW0)/[(TWt + SDW + TW0)/2 \times t] \times 100$

Daily feeding rate (%/day) = $F/[(TWt + SDW + TW0)/2 \times t] \times 100$

Feed conversion ratio (FCR) = $F/(TWt + SDW - TW0)$

Protein efficiency ratio (PER) = $(TWt + SDW - TW0)/P$

W: Body weight (g)
FL: Fork length (cm)
TW0: Initial total body weight (g)
TWt: Final total body weight (g)
SDW: Total body weight of sampled fish and dead fish (g)
Nt: Number of final fish
F: Total feed intake (g)
t: Rearing days

Table 2. Proximate composition, phosphorus content, and fatty acid composition of the experimental diets

Analysis	Diet group			
	FM	PP-SP	PP-FS	AP-SP
Proximate composition (%)				
Moisture	7.6	7.7	8.8	4.9
Crude ash	9.1	5.6	5.6	7.4
Crude protein	39.8	38.1	37.4	42.0
Crude lipid	18.9	16.1	17.4	17.6
Phosphorus content				
P (mg/g)	15.4	9.7	9.7	13.4
Fatty acid composition (%)				
14: 0[*1]	4.8	4.2	4.3	5.4
16: 0	18.3	16.5	18.3	15.8
18: 0	3.8	3.5	4.1	4.2
16: 1n-7	5.8	5.2	5.5	5.5
18: 1n-9	16.0	16.8	19.0	16.4
20: 1n-9	2.6	2.7	4.5	7.5
20: 1n-11	2.2	2.3	1.5	1.5
22: 1n-11	4.0	4.1	4.5	9.8
18: 2n-6	4.5	7.0	7.1	5.6
20: 4n-6	1.0	0.9	0.9	0.4
18: 3n-3[*2]	1.0	1.2	1.1	0.9
18: 4n-3	1.8	1.5	1.3	2.0
20: 4n-3	0.6	0.6	0.6	0.6
20: 5n-3 (EPA)	8.0	6.8	6.0	5.2
22: 5n-3 (DPA)	2.0	1.9	1.9	1.0
22: 6n-3 (DHA)	13.5	13.6	11.3	6.2
Others	10.2	11.1	8.0	12.2
n-3 HUFA	25.9	24.3	21.2	14.9

[*1] Left figure represents the number of carbonatoms and right figure after colon represents the number of carbon unsaturated bonds.

[*2] "n-3"represents the omega-3 fatty acid.

n-3 HUFA=18: 4n-3+20: 4n-3+EPA+DPA+DHA.

Experimental diets are shown in Table 1.

P: Total protein intake (g)

Blood, muscle, and liver samples were collected from 5 fish in every group at the start (initial), late summer (intermediate), and late autumn (end). The ratio of liver weight per body weight was calculated. Hematocrit value and plasma component levels were analyzed. General content, phosphorus content, fatty acid composition, and amino acid composition of muscle were analyzed.

The statistical differences between trial diets were determined by Tukey's multiple range comparison test ($p<0.05$).

Experimental diet uptake, stomach evacuate rate, and comparative disease resistance of fish fed experimental diets: Nutritional absorption was analyzed from fish feces collected from fish reared in a conical tank (Fig. 2) using special diets containing chrome oxide as an internal standard material (Satoh *et al.*, 2013).

Fish were anesthetized and administered the diet at 2% of body weight directly into the stomach. Stomach residue was analyzed over time by the Kjeldahl nitrogen method and stomach evacuation rate was estimated (Tohata *et al.*, 2012).

Disease resistance was carried out using *Streptococcus dysgalactiae*, an infectious streptococcal bacterium. Fish were reared on each diet in indoor tanks for 3 months and fed the bacterium-containing feed for 5 days. Fish mortality was observed over a 40 day period (Ishida *et al.*, 2013).

Diet palatability: Fish behavior was divided into five responses after placing the diets into a fish tank described as recognition, ingestion, orientation within the mouth, swallowing, or spitting out (Fig. 3). The actions were analyzed by using a camera to film red sea bream, yellowtail, and coho salmon using single dry pellets made from only one material (Fig. 4) (Niinuma *et al.*, 2012, Yamauchi *et al.*, 2012).

Meat quality of raw sashimi: A panel test using the order method, color-difference meter analysis, and electronic nose system analysis were performed for each experimental fish fed PP-SP, PP-FS, AP-SP, and control diet (Tohata *et al.*, 2013).

Fig. 2. The conical tank used to collect feces of fish fed the experimental diets to analyze nutritional absorption.
Right: Feces at the bottom of the tank

RESULTS

Processing: All the experimental diets were processed using a double-screw extruder. At the beginning in the autumn season, plant protein based diets did not sink at a suitable rate and flowed out from the net cage compared to the other diets. Therefore these diets were re-made. Palatability ingredients and an enzyme solution were sprayed onto diets to minimize materials used to 0.05% from the original 2% required in the blending method (Fig. 1).

Growth performances and biological indices in yellowtail: The growth performances in both the summer and autumn period are shown in Table 3.

In the summer period when the water temperature was 22.8 - 29.2 ℃ all diet groups

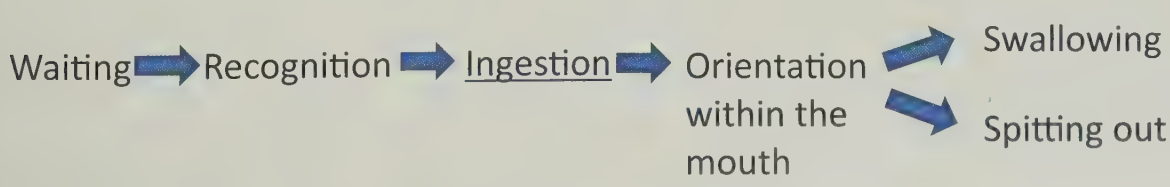


Fig. 3. Fish behaviors for the diet.

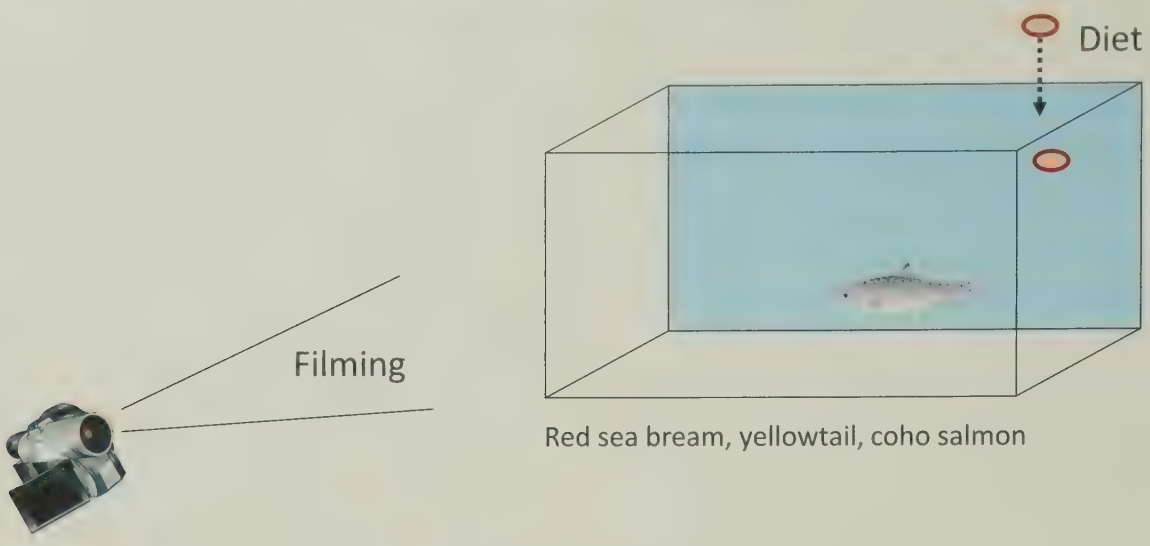


Fig. 4. Behavioral analysis of fish after feeding the diets.

Table 3. Growth performance and feed utilization by yellowtail fed the experimental diets

Rearing season (days)		Initial	Summer (90 days)				Autumn (91 days)			
			FM	PP-SP	PP-FS	AP-SP	FM	PP-SP	PP-FS	AP-SP
Number of fish		220[*1]								
	Dead		0	0	1	2	1	0	1	1
	Sampled		3	4	3	4	7	7	7	7
	Final		52	51	51	49	44	44	43	41
Total food intake (kg)		–	113.0	113.9	113.6	118.0	98.5	102.9	101.0	90.5
Body weight (g)[*2]		753 ± 96								
	Final		1663 ± 204[a]	1700 ± 221[a]	1698 ± 156[a]	1688 ± 204[a]	2242 ± 333[a]	2178 ± 285[a]	2234 ± 244[a]	2205 ± 222[a]
Fork length (cm)[*2]		36.9 ± 1.5								
	Final		44.8 ± 2.3[a]	44.8 ± 1.8[a]	45.0 ± 1.2[a]	44.1 ± 1.2[a]	48.4 ± 2.2[a]	48.6 ± 1.8[a]	49.0 ± 1.7[a]	48.5 ± 1.5[a]
Condition factor[*2]		15.0 ± 0.8								
	Final		18.3 ± 1.1[b]	18.8 ± 0.9[b]	18.6 ± 0.7[b]	19.7 ± 1.4[a]	19.6 ± 1.2[a]	18.9 ± 1.2[b]	19.0 ± 1.1[ab]	19.3 ± 1.0[ab]
Weight gain ratio (%)[*3]		–	116.4	119.9	119.1	113.6	34.2	30.4	31.0	32.3
Daily weight gain (%/day)[*3]		–	0.82	0.83	0.83	0.80	0.32	0.29	0.30	0.31
Daily feeding rate (%/day)[*3]		–	1.92	1.91	2.02	1.91	1.08	1.15	1.05	1.12
Feed conversion ratio[*3]		–	2.35	2.29	2.30	2.51	3.33	3.91	3.76	3.39
Protein efficiency ratio[*3]		–	1.07	1.14	1.16	0.94	0.75	0.67	0.71	0.70

[*1] Fishes were divided into 4 diet groups, each group contains 55 fishes.
[*2] Data are shown as mean ± standard deviation (n=20–44). Values having different superscript letters are significantly different ($P<0.05$).
[*3] Shown in the text.
Experimental diets are shown in Table 1.

showed a similar total feed intake ranging from 113 to 118 kg and a similar body weight gain (Fig. 5). In the autumn period when the water temperature was 11.5 ℃ lower than during the summer period total food intake of all groups was less than in the summer ranging from 90 to 103 kg. No seasonal difference in weight gain was seen among diet groups.

Final fork length and body weight were 48.4 cm and 2,242 g in the control (FM), 48.6 cm and 2,178 g in the PP-SP, 49.0 cm and 2,234 g in the PP-SF, and 48.5 cm and 2,205 g in the AP-SP. Differences between control and non-fish meal diets were not observed and the growth was almost the same. There was no seasonal difference between daily weight gain (%/day), daily feeding rate (%/day), and

weight gain ratio (%). For example, feed conversion ratio in the autumn season was 3.33 in the control (FM) and 3.91 in PP-SP, 3.76 in PP-FS, and 3.39 in AP-SP.

Table 4 shows the results of the biological indices of cultured fish (n=5). The liver to body weight ratio in every group was 1.2 to 1.3 and no significant difference was seen. The average hematocrit value of 5 fish in every group was 46.2 to 50.6 with no significant difference observed. There were three, two, and one mortalities of fish in AP-SP, PP-FS, and FM respectively. Green liver, which is thought to be a sign of taurine deficiency (Takagi *et al.*, 2005), was not seen in any of the dead fish.

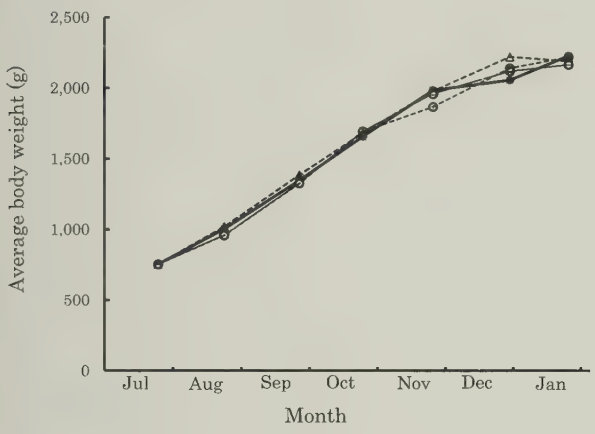


Fig. 5. Growth curves of yellowtail fed the experimental fish meal diet (FM : -●-) and non-fish meal diet (PP-SP : -○-, PP-FS : -⊖-, and AP-SP : -△-) in net cages for 6 months. Experimental diets are detailed in Table 1.

Plasma components measured included glucose, total protein, total cholesterol, glutamate pyruvate transaminase (GPL), and alkaline phosphatase (ALP). No significant difference was observed in plasma components between the treatments in the summer period. Total cholesterol exhibited a significant seasonal variability between the treatments with lower values of 246 and 266 mg/dl in PP-SP and PP-FS respectively (p<0.05) (Table 4).

Characteristics such as uptake, stomach evacuate rate, and disease resistance of fish fed the experimental diets: The uptake of protein from non-fish meal diets was reduced by 4–9 % compared to the FM control (Satoh *et al.*, 2013).

Evacuation of feed from fish stomachs was observed for all three diets. The order of the rate of evacuation was PP-SP > AP-SP ≈FM (Fig. 6).

Survival rate at 42 days after the challenge test was 80% in FM group, and 95% in PP-SP and AP-SP group (Ishida *et al.*, 2013).

Diet palatability: Fish spitting out feed was almost zero for single dry fish meal pellet. The spitting rate was higher in single dry pellet made from plant protein than those made from animal protein. To improve palatability of PP and AP pellets we washed the original materials with 80% ethanol to remove the extractive component and made each single dry pellet from those pre-treated materials. The spitting rate in single dry pellet made from pre-

Table 4. Changes in hepatosomatic index, heamatocrit value, and hematochemical characteristics in yellowtail (n=5) fed the experimental deits

Rearing season (days)	Initial	Summer (90 days)				Autumn (91 days)			
		FM	PP-SP	PP-FS	AP-SP	FM	PP-SP	PP-FS	AP-SP
Hepatosomatic index (%)*1	1.1±0.0	1.4±0.1^a	1.2±0.1^a	1.2±0.2^a	1.3±0.3^a	1.4±0.2^a	1.3±0.1^a	1.2±0.0^a	1.2±0.1^a
Hematocrit value (%)	53.6±3.4	61.5±7.4^a	50.8±0.7^a	57.0±4.9^a	55.3±7.3^a	46.2±2.1^a	45.7±4.5^a	50.6±6.5^a	48.0±6.2^a
Heamatochemical characteristics									
Glucose (mg/*dl*)	144±25.2	237.8±51.8^a	262.6±30.8^a	267.0±41.3^a	280.8±146.1^a	138.6±10.5^a	127.6±21.3^a	143.0±12.1^a	130.0±28.5^a
Total protein (g/*dl*)	4.0±0.5	4.3±0.7^a	3.7±0.2^a	4.3±1.5^a	4.8±0.7^a	4.1±0.5a^b	3.7±0.2^b	3.9±0.5a^b	4.4±0.4^a
Total cholesterol (mg/*dl*)	349.4±63.0	282.0±30.8^a	203.4±21.7^a	267.0±79.5^a	276.4±101.0^a	396.8±72.3^a	245.6±29.0^b	266.4±61.0^b	346.0±82.1^{ab}
GPT (IU/*l*)*2	25.4±2.6	32.4±4.2^a	59.2±44.1^a	34.6±14.5^a	44.4±19.9^a	32.0±13.4^a	30.8±14.8^a	23.4±8.6^a	33.8±11.4^a
ALP (IU/*l*)*3	148.6±33.8	156.4±34.3^a	134.8±14.4^a	181.0±64.0^a	200.6±22.9^a	143.4±43.4^a	125.2±16.9^a	126.5±23.7^a	147.8±42.3^a

Mean ± standard deviation (n=5). Values having different superscript letters are significantly different (P<0.05).
*1 The ratio of liver weight to body weight.
*2 Glutamate-pyruvate transaminase.
*3 Alkaline phosphates.
Experimental diets are shown in Table 1.

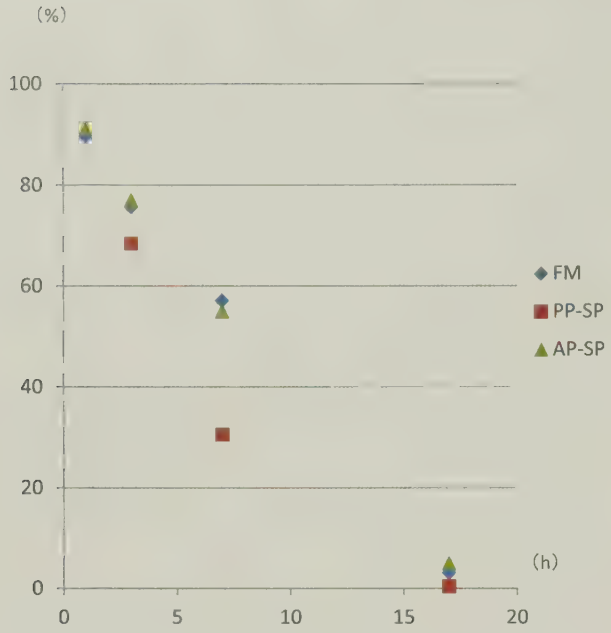


Fig. 6. Evacuation of the diet from the fish stomach after a single oral administration of diet at 2% body weight. Value are shown the percentage of nitrogen in the stomach residue per administered nitrogen. Experimental diets are shown in Table 1.

treated plant protein decreased. On the other hand, the opposite result was shown in pre-treated animal proteins that the spitting rate increased (Niinuma *et al.*, 2012, Yamauchi *et al.*, 2012).

Fish meat analysis: Table 5 shows the results of the fish meat analysis of cultured fish. In the proximate composition of all the diet groups, moisture content declined and fat content increased until the end of experiment. No difference was seen in the phosphorus content among the diet groups. The EPA, DHA and n3HUFA content of fish fed AP-SP pellets was always lower than in fish fed the other diets, at times significantly less ($p<0.05$) in both summer and autumn. These results were similar to the analysis of experimental diets.

Discussion

It has long been thought that fish meal was a necessary ingredient in fish feed. Generally, the formulated feeds for yellowtail contain about 50% fish meal. Recently, formulated feeds containing lower amounts of fish meal such as 30% have been produced as a commercial product. However a fish meal content of less than 30% would have considerable financial benefits for aquaculture enabling continued economically viable production. Therefore, we developed non-fish meal feeds and evaluated those in a field experiment with a similar aquaculture environment. We used one year old yellowtail which is an intermediate size during culturing and shows rapid growth requiring high feed costs. Experimental periods included the higher feeding activity season of summer and the lower feeding activity season of winter.

In the present study (Tsuzaki *et al.*, 2014), the plant protein based diets of PP-SP and PP-SP showed a similar daily feeding rate and daily weight gain, better feed conversion ratio and protein efficiency ratio than the control FM in summer season. On the other hand, the animal protein based diet of AP-SP showed a higher daily feeding rate and feed conversion ratio, lower protein efficiency ratio demonstrating lower protein uptake than others. We also studied uptake of nutrient in these non-fish meal diets and FM by analysis of yellowtail feces. It is clear that the uptake of protein in these non-fish meal diets were reduced by 4 – 9 % compared to the control FM.

Then, in the autumn season, the plant protein based diets of PP-SP and PP-FS showed a low performance because of the higher daily feeding rate and feed conversion ratio, lower daily weight gain. These results are thought to be due to the physical properties of the diets. Because these plant protein based diets did not sink appropriately and flowed out from the net cage compared to the other diets. From these results, more studies are needed for the improvement of uptake for animal protein based diets and adjustment of the physical characteristics for plant protein based diets. However, these results demonstrate that non-fish meal diets have the potential to support the growth of one year old

Table 5. Proximate composition, phoshorus conntent, and fatty acid composition of muscle in yellowtail (n=5) fed the experimental diets

Rearing season (days)		Summer (90 days)				Autumn (91 days)			
	Initial	FM	PP-SP	PP-FS	AP-SP	FM	PP-SP	PP-FS	AP-SP
Proximate composition (%)									
Moisture	71.9 ± 0.8	64.7 ± 1.7	64.5 ± 1.4	64.8 ± 1.7	65.6 ± 4.1	60.6 ± 4.6	60.0 ± 3.0	60.3 ± 1.3	62.5 ± 0.9
Crude ash	2.3 ± 0.5	1.3 ± 0.3	1.3 ± 0.0	1.4 ± 0.1	1.4 ± 0.1	1.4 ± 0.1	1.3 ± 0.1	1.4 ± 0.2	1.4 ± 0.1
Crude protein	22.1 ± 0.2	21.4 ± 0.6	20.3 ± 1.1	20.5 ± 0.8	20.7 ± 1.3	20.1 ± 2.3	20.5 ± 1.6	22.0 ± 1.0	22.0 ± 0.5
Crude lipid	3.7 ± 0.9	11.5 ± 2.1	12.8 ± 2.6	10.6 ± 1.2	10.1 ± 3.3	16.0 ± 6.2	17.9 ± 4.7	16.0 ± 1.2	14.3 ± 1.5
Phosphorus content									
P (mg/g)	15.5 ± 4.4	4.2 ± 1.7	5.5 ± 2.8	5.0 ± 1.5	4.3 ± 2.5	4.6 ± 1.1	4.5 ± 1.6	4.4 ± 0.7	4.7 ± 1.4
Fatty acid composition (%)									
14: 0	4.2 ± 0.4	4.0 ± 0.3	3.1 ± 0.6	3.4 ± 0.7	3.9 ± 0.2	4.3 ± 0.2	3.9 ± 0.4	3.8 ± 0.3	4.2 ± 0.2
16: 0	19.6 ± 0.9	18.0 ±0.8	16.2 ± 1.3	16.8 ± 1.1	16.1 ± 0.3	16.4 ± 0.4	16.5 ± 0.7	15.6 ± 0.6	16.0 ± 0.5
18: 0	5.6 ± 0.5	4.2 ± 0.3	4.0 ± 0.1	4.2 ± 0.5	4.5 ± 0.4	3.4 ± 0.1	3.4 ± 0.1	3.3 ± 0.1	3.9 ± 0.0
16: 1n-7	5.9 ± 0.8	6.6 ± 0.3	5.8 ± 0.5	5.9 ± 0.4	5.9 ± 0.3	7.0 ± 0.3	6.3 ± 0.2	6.2 ± 0.2	6.6 ± 0.3
18: 1n-9	21.4 ± 1.4	23.2 ± 2.2	25.0 ± 0.8	25.6 ± 1.3	25.0 ± 1.9	20.5 ± 1.9	23.5 ± 1.6	22.6 ± 2.0	22.1 ± 0.3
20: 1n-9	3.1 ± 0.8	5.1 ± 0.3	5.4 ± 0.3	5.7 ± 0.5	9.4 ± 1.4	5.6 ± 1.2	5.3 ± 0.1	5.6 ± 0.3	6.5 ± 0.2
20: 1n-11	0.6 ± 0.9	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.3 ± 0.0	0.6 ± 0.9	0.2 ± 0.0	0.2 ± 0.0	2.9 ± 0.1
22: 1n-11	2.7 ± 0.3	3.4 ± 0.4	3.7 ± 0.5	4.0 ± 0.6	8.7 ± 0.7	3.6 ± 0.6	3.1 ± 0.1	3.7 ± 0.4	6.3 ± 0.4
18: 2n-6	5.6 ± 0.2	5.8 ± 0.2	8.6 ± 0.3	8.6 ± 0.1	7.2 ± 0.2	5.3 ± 0.1	8.4 ± 0.1	8.4 ± 0.1	7.4 ± 0.2
20: 4n-6	1.2 ± 0.2	0.9 ± 0.0	0.9 ± 0.1	0.8 ± 0.1	0.5 ± 0.1	0.8 ± 0.0	0.8 ± 0.1	0.8 ± 0.0	0.4 ± 0.0
18: 3n-3	0.6 ± 0.1	1.1 ± 0.0	1.3 ± 0.1	1.2 ± 0.1	0.9 ± 0.1	1.0 ± 0.0	1.2 ± 0.0	1.2 ± 0.0	0.8 ± 0.0
18: 4n-3	0.9 ± 0.1	1.6 ± 0.1	1.3 ± 0.0	1.3 ± 0.1	1.6 ± 0.3	1.5 ± 0.1	1.3 ± 0.1	1.4 ± 0.1	1.5 ± 0.0
20: 4n-3	0.5 ± 0.0	0.9 ± 0.0	0.7 ± 0.0	0.7 ± 0.0	0.6 ± 0.1	0.7 ± 0.0	0.6 ± 0.0	0.7 ± 0.0	0.6 ± 0.0
20: 5n-3 (EPA)	5.3 ± 0.2	6.2 ± 0.5^{a}	5.3 ± 0.4^{ab}	4.8 ± 0.9^{ab}	4.6 ± 1.1^{b}	6.6 ± 0.2^{a}	5.0 ± 0.3^{bc}	5.1 ± 0.3^{b}	4.7 ± 0.1^{c}
22: 5n-3 (DPA)	2.2 ± 0.2	2.4 ± 0.1^{a}	2.2 ± 0.2^{ab}	2.1 ± 0.2^{b}	1.5 ± 0.1^{c}	2.4 ± 0.2^{a}	2.0 ± 0.1^{b}	2.1 ± 0.1^{b}	1.4 ± 0.1^{c}
22: 6n-3 (DHA)	11.4 ± 1.6	10.9^{a}	11.3 ± 1.0^{a}	9.4 ± 3.1^{a}	4.9 ± 1.2^{b}	11.4 ± 0.6^{a}	10.2 ± 0.8^{a}	10.5 ± 1.2^{a}	5.1 ± 0.5^{b}
Others	9.2 ± 2.8	5.9 ± 1.5	5.0 ± 0.3	5.2 ± 1.0	4.5 ± 2.4	8.9 ± 1.9	7.9 ± 2.0	8.5 ± 2.0	9.8 ± 0.2
n-3 HUFA	20.3 ± 1.4	21.7 ±2.5^{a}	20.9 ± 1.6^{a}	18.3 ± 4.3^{ab}	13.2 ± 2.9^{b}	22.7 ± 0.6^{a}	19.2 ± 1.2^{b}	19.8 ± 1.5^{b}	13.2 ± 0.6^{c}

Mean ± standard deviation (n=5). Values having different superscript letters are significantly different (P<0.05).
Experimental diets are shown in Table 1.
Fatty acid compositions are shown in Table 2.

yellowtail compared to the results of Takagi *et al.* (2013).

For substitute materials, plant protein material such as soy bean concentrate has been studied (Aoki *et al.*, 2000a, 2000b). We also used soy bean concentrate and confirmed its performance as a substitute protein for fish meal in the present study. In the field, it is sometimes believed that plant protein materials stay longer in the stomach than fish meal. However, in our experiment, the plant protein based diets did not stay longer among the diet groups. Fish meal based diet was the first to be evacuated from the stomach. In addition, it is sometime believed that plant protein fed yellowtail is more susceptible to epidemic disease than fish meal based diet. However, in our experiment, those phenomena were not seen (Ishida *et al.*, 2013). We also compared the stomach evacuation rate and the *in vitro* digestion using only one protein material diet, and found minimal difference (Tohata *et al.*, 2012). More studies are needed in future using low cost materials such as defatted soy bean meal, corn gluten meal and canola oil.

As for substitute animal protein materials, there are meat meal, chicken meal, feather meal, and pork meal. Feather meal and meat meal were reported to be useful for yellowtail (Shimeno *et al.*, 1993a, 1993b, 2000). However, the animal protein studies are fewer than plant protein studies. There is potential to use chicken meal and pork meal. As there are few studies about pork meal, we tried to use pork meal in the present study. Pork meal has less DHA, DPA and n3HUFA of fatty acid than FM. A similar tendency was seen in the whole fish body and the meat of yellowtail fed by pork meal based diet. However, there is no significant effect on growth and

survival. Only slightly less protein digestion in pork meal based diet was seen than the plant protein based diet. And feeding activity on pork meal based diets was active. From these results, pork meal was thought to be available as a substitute material.

The biggest problem has been shown to be the reduction of feeding activity when fish are fed low fish meal content diets (Aoki *et al.*, 2000a, 2000b, 2000c). However, it is reported that an added palatability ingredients improved the feeding activity in eel (Takii *et al.*, 1984), yellowtail (Takii *et al.*, 1994), and tiger puffer (Takii *et al.*, 1998). In the present study, we found that fish did not especially prefer plant protein to animal protein and fish meal. However, after washing the feed ingredients to remove the extractive component, the spitting ratio decreased in plant ingredient feeds and increased in animal protein feeds, which suggested that the extractive components might affect feeding activity (Niinuma *et al.*, 2012, Yamauchi *et al.*, 2012). Then, we added some palatability ingredients to the plant based diet, fish swallowing ratio was increased and spitting ratio was decreased. From these results, we used the skipjack peptide (SP) and fish sauce (FS) as palatability ingredients in order to improve the feeding activity. In the results, higher palatability was seen when using both ingredients. The biggest problem of reduction of feeding activity in non-fish meal diets was thought to be almost resolved by the addition of palatability ingredients.

The meat quality of yellowtail fed with non-fish meal diets were compared to control yellowtail by a panel test, which showed a difference in color and smell. Then we used colorimeter and Electronic Nose System of Alpha MOS (http://www.alpha-mos.co.jp/products/sensory.html). The fish fed by non-fish meal diets had whiter muscle and a less fishy smell (Tohata *et al.*, 2013). Now, the effect of non-fish meal diets on fish meat quality and as a method of enhancing the meat quality control is being determined.

As described above, we alternated fish meal to plant or animal protein materials to make non-fish meal diets, and fed them to one year old yellowtail, and got similar growth, body and blood compositions. The main reason for the similar growth rates was thought to be the addition of palatability ingredients and enzyme mixtures (Table 1). As we used both plant and animal materials, we suggest the possibility of many materials for non-fish meal diets. More study is needed to improve low cost diets and produce higher quality meat.

This work was supported by Research and Development Projects for application in promoting new policy of agriculture, forestry and fisheries from the Ministry of Agriculture, Forestry and Fisheries of Japan.

References

Aoki H., Sanada Y., Furuichi M., Kimoto R., Maita M., Akimoto A., Yamagata Y., and Watanabe T., 2000a: Partial or complete replacement of fish meal by alternate protein source in diets for yellowtail and red sea bream. *Aquaculture Sci.*, **48**, 53–63.

Aoki H., Watanabe K., and Satoh S., 2000b: Use of low non-fish meal diets for red sea bream. *Aquaculture Sci.*, **48**, 73–79.

Aoki H., Sanada Y., Furuichi M., Watanabe K., Satoh S., Yamagata Y., and Watanabe T., 2000c: Use of non-fish meal diets for yellowtail. *Aquaculture Sci.*, **48**, 65–72.

Ishida N., Maeno K., Yanagi S., Koshiishi T., and Satoh S., 2013: Feeding experiment in room tanks of the yellowtail *Seriola quinqueradiata* with non-fish meal diet. *Program and Abstracts of the Japanese Society of Fisheries Science Spring Meeting 2013*. pp131 (in Japanese).

Niinuma T., Yamauchi A., Nakano T., Yamaguchi T., Satoh M., Katayama S., Aoyama T., Udagawa T., and Ishida N., 2012: Palatability improvement effect of vegetable protein feed by the addition of material attractive to the target species. *Program and Abstracts of the Japanese Society of Fisheries Science Autumn Meeting 2012*. pp45 (in Japanese).

Satoh S., Abe Y., Haga Y., Matsukura K., Koshiishi T., and Ishida N., 2013: Digestibility and absorption of nutrient in low/non-fish meal feed for marine fish. *Program and Abstracts of the Japanese Society of Fisheries Science Autumn Meeting 2013*. pp34 (in Japanese).

Shimeno S., Matsumoto T., Hujita T., Mima T., and

Ueno S., 1993a: Alternative protein sources for fish meal in diets of young yellowtail. *Nippon Suisan Gakkaishi*, **59**, 137-143.

Shimeno S., Mima T., Imanaga T., and Tomaru K., 1993b: Inclusion of combination of defatted soybean meal, meat meal, and corn gluten meal to yellowtail diets. *Nippon Suisan Gakkaishi*, **59**, 1889-1895.

Shimeno S., Matsumoto M., Hosokawa H., Matsumoto T., and Ukawa M., 2000: Inclusion of poultry feather meal in diet for fingerling yellowtail. *Aquaculture Sci.*, **48**, 99-104.

Takagi S., Murata H., Goto T., Ichiki T., Munasinghe D. MS, Endo M., Matsumoto T., Sakurai A., Hatae H., Yoshida T., Sakai T., Yamashita H., Ukawa M., and Kuramoto T., 2005: The Green Liver syndrome is caused by taurine deficiency in yellowtail, *Seriola quinqueradiata* fed diets without fishmeal. *Aquaculture Sci.*, **53**, 279-290.

Takagi S., Murata H., Goto T., Hatae H., Yamashita H., Takano A., and the late Ukawa M., 2013: Long-term feeding of the yellowtail *Seriola quinqueradiata* with soy protein concentrate-based non-fishmeal diet supplemented with taurine. *Aquaculture Sci.*, **61**, 349-358.

Takii K., Takeda M., and Nakao Y., 1984: Effects of supplement of feeding stimulants to formulated feeds on feeding activity and growth of juvenile eel. *Bull. Japan. Soc. Sci. Fish.*, **50**, 1039-1043.

Takii K., Shimeno S., Akutsu M., and Takeda M., 1994: Dietary supplement of feeding stimulants on performance and digestive function of yellowtail, *Seriola quinqueradiata*. *Bull. Fish. Lab. Kinki Univ.*, **4**, 127-137.

Takii K., Shimeno S., Akutsu M., and Takeda M., 1998: Dietary supplement of feeding stimulants growth and digestive enzyme secretion in Tiger puffer. *Bull. Fish. Lab. Kinki Univ.*, **6**, 159-166.

Tohata K., Koshiishi T., Satoh S., and Ishida N., 2012: *In vivo* and *in vitro* digestibleness of extruded diets of non-fish meal in red sea bream. *Program and Abstracts of the Japanese Society of Fisheries Science Autumn Meeting 2012*. pp112 (in Japanese).

Tohata K., Ishida N., Koshiishi T., Satoh S., Hotta T., Nakagawa M., Yoshida K., Tsusaki T., and Miyazaki T., 2013: Evaluation of meat quality of yellow tail fed by extruded diets of non-fish meal. *Program and Abstracts of the Japanese Society of Fisheries Science Autumn Meeting 2013*. pp 43 (in Japanese).

Tsuzaki T., Hotta T., Yoshida K., Nakagawa M., Koshiishi T., Yamada M., Satoh S., and Ishida N., 2013: Feeding experiment at sea of the yellowtail *Seriola quinqueradiata* with non-fish meal diet. *Program and Abstracts of the Japanese Society of Fisheries Science Spring Meeting 2013*. pp131 (in Japanese).

Tsuzaki T., Yoshida K., Hotta T., Nakagawa M., Yamada M., Satoh S., Koshiishi T., Maeno K., Akiyama T., and Ishida N., 2015: Long-term experimental feeding of yellowtail *Seriola quinqueradiata* with complete non-fish meal extruded-pellet (EP) diets. *Aquaculture Sci.*, **63**, 29-38.

Yamauchi A., Niinuma A., Nakano T., Yamaguchi T., Satoh M., Katayama S., and Ishida N., 2012: Palatability evaluation of alternative fish feed ingredients using coho salmon. *Program and Abstracts of the Japanese Society of Fisheries Science Autumn Meeting 2012*. pp45 (in Japanese).

水研センター研報，第40号，51－59，平成27年
Bull. Fish. Res. Agen. No. 40, 51－59, 2015

The Importance of Taurine and n-3 Fatty Acids in Cobia, *Rachycentron canadum*, Nutrition

Aaron M. WATSON[*1], Frederic T. BARROWS[*2], and Allen R. PLACE[*3]

Abstract: In the wild, fish can adapt to their diet by down regulating or eliminating biosynthetic pathways for nutrients found in abundance in their prey. Upon domestication for aquaculture these fish may now have unique dietary requirements which must be met through supplementation. To examine this hypothesis and aid in determining potential minimum requirement levels for aquaculture production, we have examined the synthetic capacity for taurine and polyunsatured fatty acids in cobia, *Rachycentron canadum*, through molecular methods and growth trials, respectively.

Taurine, a non-protein amino acid, is found in high concentrations in the natural diet of cobia and plays a variety of physiological roles. The taurine biosynthesis pathway in vertebrates is well known; however it appears to be nonfunctional in some terrestrial (Felidae) and marine (Rachycentridae) carnivores, thereby necessitating dietary supplementation. Taurine is not found in plant protein sources used for replacing fishmeal in aquaculture feeds, an increasingly important priority.

We examined the effects of graded levels of taurine addition (0%, 0.5%, 1.5%, 5.0%) to plant protein and fishmeal based diets formulated and manufactured by the USDA-ARS. At the conclusion of a feeding trial, RNA was extracted from liver, muscle, and brain tissue for quantitative-RT-PCR analysis of the genes involved in taurine synthesis. Cysteine dioxygenase (CDO), cysteamine dioxygenase (ADO), and taurine transporter (TauT) activity and expression levels were examined and no differences in transcript abundance was detected within the tissues between the dietary taurine levels. Increasing dietary taurine resulted in increased tissue taurine concentrations.

To examine the effects of alternative sources completely replacing fish oil, two replacements were examined as the lipid sources in a fishmeal free, plant based feed (USDA-ARS) and compared to a fish oil version of the diet. A thraustochytrid meal plus soybean oil (TM+SOY) and a canola oil with exogenous docosahexaenoic (DHA) and arachidonic (ARA) acids (CO+EFA) were utilized. At the conclusion of an eight week growth trial, whole body and fillet fatty acid profiles were examined.

The TM+SOY diet worked equivalently to the USDA-ARS control diet, however the CO+EFA diet resulted in significantly lower growth and survival as well as an increased feed conversion ratio. Whole body fatty acid profiles revealed a significant reduction in total essential fatty acid (EFA) concentration in the CO+EFA fed fish, indicating the supplemented levels of DHA and ARA were insufficient to meet requirements. Although all three diets were sufficient in the precursors for EFA synthesis, this reduction in growth, survival, and whole body EFA concentration suggests cobia have limited synthetic capacity for DHA and ARA. Both alternative lipid diets were devoid of eicosapentanoic acid (EPA) supplementation, which does not appear to be essential for cobia as the TM+SOY diet performed equivalently to the fish oil control.

Key words: Cobia, Taurine, Docosahexaenoic Acid, Fishmeal and Fish Oil Replacement

2015年1月30日受理（Received on January 30, 2015）

*1 Assistant Marine Scientist, Marine Resources Research Institute, South Carolina Department of Natural Resources E-mail: WatsonA@dnr.sc.gov
*2 U.S. Department of Agriculture, Agricultural Research Service
*3 Institute of Marine and Environmental Technology, University of Maryland Center for Environmental Science

World fish supply has grown on average 3.2 percent per year over the last five decades, with much of the growth occurring in various aquaculture sectors (FAO, 2012). With worldwide capture fisheries holding relatively static over the last few decades, and expected to remain at current levels, aquaculture must continue to expand rapidly to meet the protein demands of the ever increasing world population. Traditionally, fishmeal and fish oil acquired through reduction of wild capture fisheries have served as major protein and lipid sources, respectively, for compounded fish feeds utilized for many species in culture. In order for aquaculture to continue to grow at current and desired rates, the inclusion levels of these ingredients must be continually reduced or eliminated, as their abundance is finite. The reduction of fishmeal and fish oil use as well as alternate protein and lipid sources have been studied extensively in many species and has been established as a priority for research efforts and the aquaculture industry as a whole (Rust *et al.*, 2011).

Reductions in productivity including decreased growth rates, increased feed conversion ratios, low feed intake, and negative health impacts have been observed in many species when reducing fishmeal below species specific threshold levels (Glencross *et al.*, 2011; Kader *et al.*, 2012; Luo *et al.*, 2006; Thiessen *et al.*, 2004; Wacyk *et al.*, 2012; Xie *et al.*, 1998). This is often related to deficiencies in amino acids, fatty acids, vitamins, or minerals that are not present in sufficient quantities in sources utilized as fishmeal replacements. This is especially true when utilizing plant proteins (wheat flour, corn gluten, soybean meal, barley, etc) as plants have substantially different amino acid profiles when compared to fishmeals or other meat based protein meals. Many of these deficiencies have been identified (lysine, methionine, threonine) and can be corrected for by simple crystalline amino acid addition to feed formulations. One challenge brought about by reducing fishmeal in favor of plant proteins is in identifying deficiencies on a species specific basis and then determining minimal and optimal supplementation levels. Similarly, when reducing or replacing fish oil in favor of alternative lipid sources, some specific fatty acids critical to growth and health are lacking in terrestrial oil sources when compared to fish oil.

Taurine, a non-protein amino acid, is found in high concentrations in fishmeal, wild prey items, and meat based meals but is devoid from plant protein sources. Taurine plays multiple important physiological roles as an osmolyte, antioxidant, photoreceptor protectant, and as a bile salt conjugate (Schuller-Levis and Park, 2003). Multiple studies have shown advantages to supplementing taurine when reducing fishmeal in a variety of species (Chatzifotis *et al.*, 2008; Kim *et al.*, 2005, 2003; Lunger *et al.*, 2007; Pinto *et al.*, 2010).

Long chain polyunsaturated fatty acids, such as docosahexaenoic (DHA) and arachidonic (ARA) acids are also not typically observed in terrestrial oils, and the ability of teleost species to convert precursors into these important fatty acids is typically low and highly variable, rendering them essential for many marine species. As with taurine and several other compounds, these fatty acids in particular must be supplemented to feeds utilizing alternative protein or lipid sources.

Cobia, *Rachycentron canadum*, is a fast growing marine carnivore whose natural diet is high in fish and crabs (Arendt *et al.*, 2001; Franks *et al.*, 1996) and is therefore likely sufficient in both taurine and the essential fatty acids DHA and ARA. Cobia is a promising species for intensive aquaculture as they can be spawned and maintained in captivity, are highly fecund serial spawners, and have high quality flesh suitable for multiple consumer types (Holt *et al.*, 2007). A great deal of research has focused on cobia nutrition and reducing and replacing both fishmeal and fish oil with alternative sources to develop sustainable feeds (Lunger *et al.*, 2006; Salze *et al.*, 2010; Trushenski *et al.*, 2012; Watson *et al.*, 2012).

Here, several studies were carried out with juvenile cobia to determine the potential synthetic capacity for taurine present in various tissues and to evaluate two fishmeal and fish oil free diets.

Materials and Methods

To examine the effects of dietary taurine on growth and performance, as well as the taurine synthetic capacity of juvenile cobia, four diets

Table 1. Dietary formulations for the diets with graded levels of taurine. From Watson *et al.* 2014.

Ingredient (g kg^{-1})	FM1	FM2	FM3	FM4
Menhaden Fish Meal	345	345	345	345
Corn Protein concentrate	44.3	44.3	44.3	44.3
Poultry by-product meal	118	118	118	118
Wheat Flour	242.7	237.7	227.7	192.7
Soybean meal, solvent extracted	90	90	90	90
Blood meal, spray dehydrated	39	39	39	39
Menhaden Fish Oil	90	90	90	90
Vitamin Pre-mix[1]	20	20	20	20
Choline CL	6	6	6	6
Stay-C	2	2	2	2
Trace mineral pre-mix[2]	1	1	1	1
Mycozorb	2	2	2	2
Taurine	0	5	15	50

[1] Contributed per kg diet; vitamin A, 9650 IU; vitamin D, 6.6 IU; vitamin E, 132 IU; menadione sodium bisulfite, 4.7 mg; thiamine mononitrate, 9.1 mg; riboflavin, 9.6 mg; pyridoxine hydrochloride, 13.7 mg; pantothenate, DL-calcium, 101.1 mg; cyanocobalamine, 0.03 mg; nictonic acid, 21.8 mg; biotin, 0.33 mg; folic acid, 2.5 mg.

[2] Contributed in mg kg^{-1} of diet; zinc 37; manganese, 10; iodine, 5; copper, 1.

were formulated to contain 0.0%, 0.5%, 1.5%, and 5.0% supplemental taurine to a fishmeal based diet (Table 1). Juvenile cobia (~125g) were housed in a recirculating aquaculture system at the Institute of Marine and Environmental Technology in Baltimore, Maryland, USA and fed for 8 weeks. At the conclusion of the eight week feeding trial, growth and feed conversion ratios were calculated, and tissues (muscle, liver, and brain) were preserved in RNA later for quantitative-PCR analysis of several genes involved in taurine synthesis. Plasma, liver, and muscle tissues were also taken for taurine content analysis. Full materials and methods are available in Watson *et al.* (2014). A parallel study was conducted with a fishmeal free, plant protein based formulation also containing 0.0%, 0.5%, 1.5%, and 5.0% supplemental taurine and similar analyses were conducted at the conclusion of the trial.

To examine the effects of fish oil replacement in a fishmeal free, plant protein based diet similar methods were employed. Two experimental diets were formulated and manufactured by the USDA-ARS (Table 2). One experimental diet utilized canola oil plus exogenous DHA (~2.85 g kg^{-1}) and ARA (~0.32 g kg^{-1}) (CO+EFA diet) while the other experimental diet utilized a thraustochytrid meal plus soybean oil (TM+SOY) as the lipid source. The control diet consisted of the same plant protein base as the experimental diets, but utilized fish oil as the lipid source. An eight week growth trial was conducted beginning with ~130g juvenile cobia and at the conclusion growth and feed conversion ratio were calculated, and fatty acid profiles of fillets were determined through gas chromatography (GC). Full materials and methods are available in Watson *et al.*, 2013a.

Results

Full results of both studies are available in the cited references, only a brief synopsis is presented here. Taurine supplementation to a fishmeal (~35%) diet did not have significant effects on growth rate, protein efficiency ratio, total bile salt concentration, or feed conversion ratio (Table 3). Transcript abundances of CDO, ADO, and TauT were also not affected by dietary taurine input level in any of the tissues examined (Fig. 1), however the rate limiting step of the reaction, cysteinesulfinate decarboxylase (CSD) was not detected through the q-PCR methods utilized. Tissue taurine levels for plasma, liver, and muscle did increase with increasing dietary taurine.

In contrast, taurine supplementation to the plant protein based diet did have significant effects on growth rate and feed conversion ratio although overall growth was significantly lower in all treatments of the plant protein formulation when compared to the fishmeal based series (Table 4). Similarly to the fishmeal series, transcript abundances for CDO, ADO, and TauT were unaffected by dietary taurine input (Fig. 1).

In the fish oil replacement study, the TM+SOY diet performed similarly to the ARS control fish oil diet while the CO+EFA diet significantly underperformed in regards to growth rate, feed conversion ratio, and survival (Table 5; Watson *et al.*, 2013a). The different lipid sources utilized in the diets did affect whole body and fillet lipid profiles, with direct similarity to the profiles of the diets (Fig. 2; Watson *et al.*, 2013a). The alternate lipid

Table 2. Diet formulations and fatty acid compositions of the diets used for fish oil replacement. FromWatson *et al.*, 2013a.

	Diet		
Ingredient (g kg^{-1})	**TM+SOY**	**CAN+EFA**	**ARS Control**
Soy Protein Concentrate	269.3	269.3	269.3
Corn Gluten	211	211	211
Wheat Flour	226.5	226.5	226.5
Soybean Meal			
Solvent Extracted	121	121	121
Menhaden Oil	0	0	84
Soybean Oil	5	0	0
Algamac 3050	79	0	0
Canola Oil + DHA + ARA	0	84	0
Dicalcium Phosphate	23.7	23.7	23.7
Vitamin Pre-mix[1]	10	10	10
Lysine-HCL	15.5	15.5	15.5
Choline CL	6	6	6
Trace Mineral Pre-mix[2]	1	1	1
Magnesium Oxide	0.5	0.5	0.5
Stay-C	3	3	3
DL-Methionine	5.8	5.8	5.8
Threonine	2.1	2.1	2.1
Potassium Chloride	5.6	5.6	5.6
Taurine	15	15	15
Fatty Acid (g $100g^{-1}$)[1]			
12:0	0.15	0.34	0.10
14:0	4.33	1.21	4.88
16:0	17.17	7.73	22.89
17:0	0.16	0.15	0.47
18:0	2.77	3.49	5.31
20:0	0.28	0.82	0.30
22:0	0.24	0.38	0.14
SFA[3]	25.10	14.12	34.08
16:1*n*-7	0.23	0.46	5.44
18:1*n*-7	0.87	2.58	2.46
18:1*n*-9+6	13.86	48.28	19.53
20:1*n*-15+cis-8	0.06	0.03	0.22
20:1*n*-9	0.14	0.76	1.21
24:1*n*-9	0.0	0.13	0.32
MUFA[4]	15.15	52.24	29.18
16:3*n*-4	0.05	0.06	0.57
16:4*n*-1	0.04	0.06	0.79
18:2*n*-6	33.27	22.86	12.74
18:3*n*-3	3.94	6.25	1.19
20:2*n*-6	0.04	0.13	0.18
20:4*n*-6	0.70	0.32	0.84
20:5*n*-3	0.42	0.27	8.90
22:5*n*-6	6.09	0.66	0.31
22:5*n*-3	0.16	0.16	1.51
22:6*n*-3	14.93	2.85	8.57
PUFA[5]	59.75	33.64	36.75
n-3[6]	19.45	9.53	20.17
n-6[7]	40.10	23.97	14.07
n-3:*n*-6	0.49	0.40	1.43

[1]Contributed per kg diet; vitamin A, 13510 IU; vitamin D, 9.2 IU; vitamin E, 184.4 IU; menadione sodium bisulfite, 6.6 mg; thiamine mononitrate, 12.7 mg; riboflavin, 13.4 mg; pyridoxine hydrochloride, 19.2 mg; pantothenate, DL-calcium, 141.5 mg; cyanocobalamine, 0.04 mg; nictonic acid, 30.5 mg; biotin, 0.46 mg; folic acid, 3.5 mg.
[2] Contributed in mg kg^{-1} of diet; zinc 37; manganese, 10; iodine, 5; copper, 1.
[3] Saturated fatty acids = sum of all fatty acids without double bonds.
[4] Monounsaturated fatty acids = sum of all fatty acids with a single double bond.
[5] Polyunsaturated fatty acids = sum of all fatty acids with two or more double bonds.
[6] Sum of all n-3 fatty acids.
[7] Sum of all n-6 fatty acids

Table 3. Performance characteristics from the graded taurinein a fishmeal diet growth trial. Within a row, different letters indicate significant differences ($P<0.05$). From Watson *et al.* 2014.

Diet	FM1	FM2	FM3	FM4
Initial Weight (mean ± SD)	127.44 ± 13.31	122.70 ± 14.97	130.64 ± 12.37	127.36 ± 14.52
Survival (%)	100	100	100	100
Weight Gain (g fish^{-1})[1]	227.83 ± 18.15	297.62 ± 24.20	313.47 ± 31.36	290.08 ± 33.11
FCR[2]	1.52 ± 0.10	1.42 ± 0.02	1.43 ± 0.11	1.51 ± 0.05
SGR[3]	2.1 ± 0.12	2.2 ± 0.04	2.2 ± 0.14	2.1 ± 0.09
PER[4]	1.35 ± 0.15	1.44 ± 0.04	1.26 ± 0.43	1.29 ± 0.07
HSI[5]	3.14 ± 0.14	2.99 ± 0.18	2.78 ± 0.45	2.72 ± 0.19
Total Bile Salts (mM)	28.48 ± 4.79	32.69 ± 6.04	30.02 ± 4.34	29.59 ± 2.32

[1]Weight Gain (g fish) = (final weight (avg per fish) − initial weight (avg per fish)

[2] Feed Conversion Ratio (FCR) $= \left(\frac{\text{food fed (g)}}{\text{weight gained (g)}}\right)$

[3]Specific Growth Rate (SGR) $= 100 * \left(\frac{\ln \text{final weight (g)} - \ln \text{initial weight(g)}}{\text{days of growth trial}}\right)$

[4]Protein Efficiency Ratio (PER) $= \left(\frac{\text{weight gained (g)}}{\text{protein fed (g)}}\right)$

[5]Hepatosomatic Index (HSI) $= 100 * \left(\frac{\text{liver weight (g)}}{\text{body weight (g)}}\right)$

Table 4. Performance characteristics from the graded taurine in a plant based diet growth trial (128.37 g initial weight). Within a row, values that share common superscripts are not significantly different from one another ($P>0.05$).

Diet (Taurine %)	PP1 (0.02)	PP2 (0.39)	PP3 (1.35)	PP4 (4.08)
Weight Gain (%)	23.35 ± 22.82^{a}	80.57 ± 60.24^{a}	130.87 ± 25.20^{b}	133.82 ± 10.83^{b}
FCR[1]	6.38 ± 1.49^{a}	2.97 ± 1.17^{b}	1.98 ± 0.19^{b}	2.12 ± 0.23^{b}
SGR[2]	0.57 ± 0.12^{a}	1.31 ± 0.26^{b}	1.47 ± 0.19^{b}	1.51 ± 0.09^{b}
Total Bile Salts (mM)	38.76 ± 7.40	28.37 ± 1.94	36.30 ± 5.69	28.75 ± 3.22

[1] Feed Conversion Ratio (FCR) $= \left(\frac{\text{food fed (g)}}{\text{weight gained (g)}}\right)$

[2]Specific Growth Rate (SGR) $= 100 * \left(\frac{\ln \text{final weight (g)} - \ln \text{initial weight(g)}}{\text{days of growth trial}}\right)$

Table 5. Production characteristics from the eight week grow out trial with fish oil replacement sources. Data from a previous grow out with juvenile cobia on a fish meal and fish oil based, commercially produced diet are included to show overall effects of fish meal and fish oil replacement. From Watson *et al.*, 2013a.

	TM+SOY	CAN+EFA	ARS Control	Commercial Diet (2009 grow out data)[6]
Weight Gain (%)[1]	288^{a}	117^{b}	275^{a}	218
Fillet Yield (%)[2]	25.93 ± 3.34	20.88 ± 4.34	25.71 ± 1.90	20.12 ± 0.01
FCR[3]	1.42^{a}	2.98^{b}	1.46^{a}	1.85
Plasma Osmolality	358.25 ± 30.59^{a}	311.75 ± 36.82^{b}	327.58 ± 13.83^{a,b}	na
PCB Content (ng g^{-1})	8.40 ± 1.54	9.30 ± 3.41	13.2 ± 4.2	45.5 ± 3.8
Mercury Content(ng g^{-1})	30.20 ± 3.53	48.17 ± 16.24	20.14 ± 2.96	71.37 ± 3.56
Hepatosomatic index[4]	1.83 ± 0.19^{a}	2.93 ± 0.79^{b}	2.66 ± 0.55^{a,b}	3.15 ± 0.002
Specific Growth Rate[5]	2.48^{a}	1.42^{b}	2.36^{a}	1.93
Survival	100%^{b}	58%^{a}	98%^{b}	100%

[1] Weight gain=(final tank weight - initial tank weight)/ initial tank weight*100.

[2] Fillet yield=(fillet weight/body weight)*100.

[3] FCR=Feed conversion ratio= grams fed/grams gained.

[4]Hepatomsomatic index =liver weight/ body weight.

[5] SGR=specific growth rate= $((\ln BW_2 - \ln BW_1)*(\text{days of growth trial}^{-1}))*100$.

[6] Initital Weight 120g, eight week growth trial (Watson *et al.* 2012).

Values in the same row with different superscripts are significantly different ($p<0.05$), no superscript indicates no significant difference within a category.

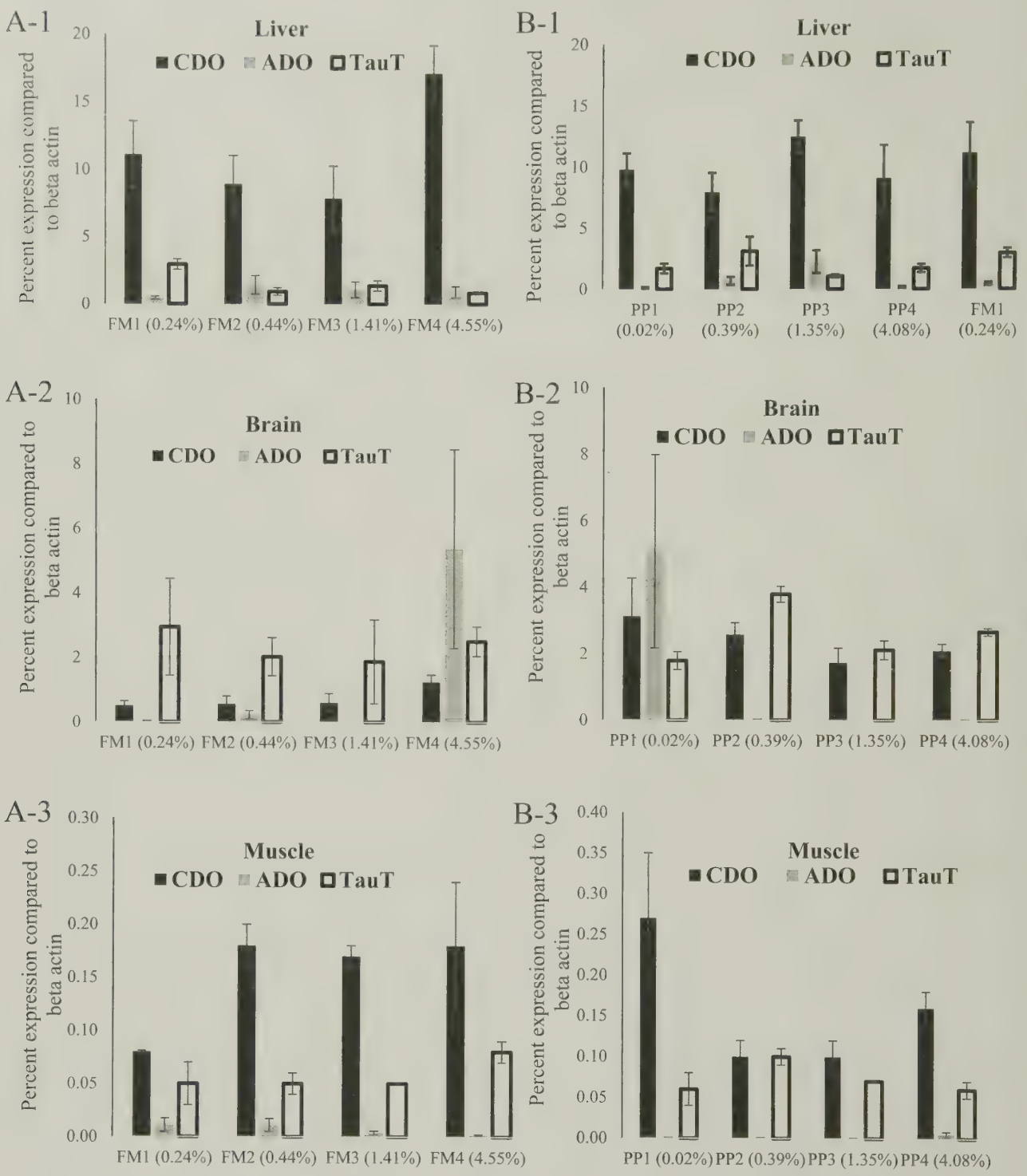


Fig. 1. Relative transcript expression (mean ±SE) of genes involved in taurine synthesis and transport in juvenile cobia, *Rachycentron canadum*, as a percent expression compared to reference gene beta-actin for each diet (% taurine) in liver (1), brain (2), and muscle (3). Equivalent cDNA input (10 ng) for triplicate samples of each tissue and six fish sampled per treatment group and tissue (n = 6 per data point). Numbers in parenthesis indicate measured taurine in each diet. Panels A-1, A-2, and A-3 depict results from Watson *et al.* (2014). with fishmeal based diet series and panels B-1, B-2, and B-3 depict results from fishmeal free, plant protein based diet series.

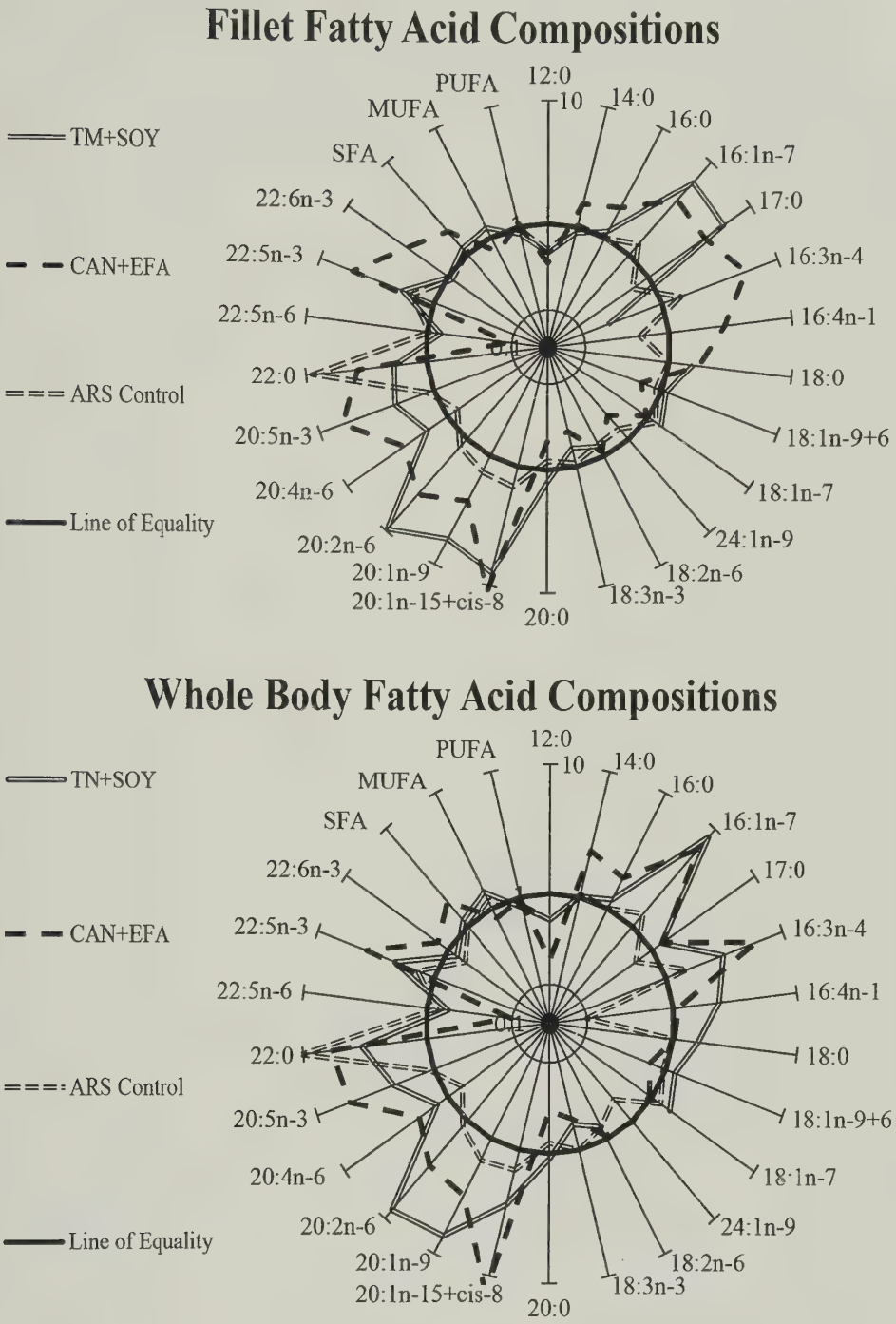


Fig. 2. Fatty acid compositions of fillet and whole body tissues from juvenile cobia fed experimental fish oil replacement diets expressed as a fraction of dietary total lipid profile. Values were calculated from relative fatty acid methyl ester (FAME) composition (Fillet or whole body fatty acid concentration/Diet fatty acid concentration). Based on this calculation, a value of 1 represents equality between fillet and dietary fatty acid composition. From Watson *et al.*, 2013a.

source diets also resulted in lower total lipid content of the fillets with the TM+SOY diet resulting in 5.81 ± 0.77% lipid, the CO+EFA diet resulting in 5.53 ± 3.15% lipid, and the control ARS fish oil diet resulting in 7.90 ± 1.75% lipid.

Discussion

These studies illustrate the importance of determining species specific requirement levels for compounds which need to be supplemented separately to feeds. Results from the taurine supplementation to a fishmeal based diet indicate that diets containing at least 35% fishmeal contain sufficient taurine and do not require additional supplementation. However, the trial with a fishmeal free, plant protein based formulation reveal negative effects of low taurine such as low growth and high feed conversion, which are alleviated by increasing dietary taurine, up to ~5.0% supplementation. These effects, combined with the lack of modulation at the transcript level of genes involved in potential taurine synthesis and transport appear to indicate that juvenile cobia are unable to synthesize taurine in sufficient quantities, if at all. Taurine should therefore be considered essential, especially when reducing fishmeal in favor of plant based proteins.

DHA and ARA levels were most likely insufficient in the CO+EFA diet to meet the nutritional requirements of juvenile cobia. This also potentially indicates limited or no synthetic capacity for these specific fatty acids in this species. Gilthead sea bream, *Sparus aurata*, in contrast performed equivalently on all three of the exact same diets from this study (Watson *et al.*, 2013b). The difference in performance of these two species on the same diets could highlight the difference in requirement levels for these fatty acids between species, or different synthetic or conversion capabilities between these two carnivores.

Taken together, these studies demonstrate the ability to produce fishmeal and fish oil free diets for a fast growing, high value marine carnivore without significant loss of production characteristics. Targeted supplementation of specific compounds found to be lacking in fishmeal and fish oil replacement sources can alleviate deficiencies. This type of work will allow for the aquaculture industry to continue to expand to meet growing global needs. Species specific work also has the potential to produce optimal diets which not only maximize production but reduce losses due to waste. These two characteristics, high production and low waste, will be critical in expanding aquaculture operations of various scales and intensity while maintaining profitability around the world as global priorities shift to food and water supply safety and the environmental impacts of aquaculture.

References

Arendt M. D., Olney J. E., and Lucy J. A., 2001: Stomach content analysis of cobia, *Rachycentron canadum* from lower Chesapeake Bay. *Fish. Bull.*, **99**, 665-670.

Chatzifotis S., Polemitou I., Divanach P., and Antonopoulou E., 2008: Effect of dietary taurine supplementation on growth performance and bile salt activated lipase activity of common dentex, *Dentex dentex*, fed a fish meal/soy protein concentrate-based diet. *Aquacult.*, **275**, 201-208.

FAO, 2012: The State of World Fisheries and Aquaculture. food Agric. Organ. United Nations Rome, Italy.

Franks J. S., Garber N. M., and Warren J. R., 1996: Stomach contents of juvenile cobia, *Rachycentron canadum*, from the northern Gulf of Mexico. *Fish. Bull.*, **94**, 374-380.

Glencross B., Rutherford N., and Jones B., 2011: Evaluating options for fishmeal replacement in diets for juvenile barramundi (*Lates calcarifer*). *Aquac. Nutr.*, **17**, 722-732.

Holt G. J., Faulk C. K., and Schwarz M. H., 2007: A review of the larviculture of cobia *Rachycentron canadum*, a warm water marine fish. *Aquacult.*, **268**, 181-187.

Kader M. A., Bulbul M., Koshio S., Ishikawa M., Yokoyama S., Nguyen B. T., and Komilus C. F., 2012: Effect of complete replacement of fishmeal by dehulled soybean meal with crude attractants supplementation in diets for red sea bream, *Pagrus major*. *Aquacult.*, **350-353**, 109-116.

Kim S., Takeuchi T., Akimoto A., Furuita H., Yamamoto T., Yokoyama M., and Murata Y., 2005: Effect of taurine supplemented practical diet on growth performance and taurine contents in whole body and tissues of juvenile Japanese flounder *Paralichthys olivaceus*. *Fish. Sci.*, **71**, 627-632.

Kim S., Takeuchi T., Yokoyama M., and Murata Y., 2003: Effect of dietary supplementation with taurine, b-alanine and GABA on the growth of juvenile and fingerling Japanese flounder *Paralichthys olivaceus*. *Fish. Sci.*, **69**, 242-248.

Lunger A. N., Craig S. R., and McLean E., 2006: Replacement of fish meal in cobia (*Rachycentron canadum*) diets using an organically certified protein. *Aquacult.*, **257**, 393-399.

Lunger A. N., McLean E., Gaylord T. G., Kuhn D., and Craig S. R., 2007: Taurine supplementation to alternative dietary proteins used in fish meal replacement enhances growth of juvenile cobia (*Rachycentron canadum*). *Aquacult.*, **271**, 401-410.

Luo L., Xue M., Wu X., Cai X., Cao H., and Liang Y., 2006: Partial or total replacement of fishmeal by solvent-extracted cottonseed meal in diets for juvenile rainbow trout (*Oncorhynchus mykiss*). *Aquac. Nutr.*, **12**, 418-424.

Pinto W., Figueira L., Ribeiro L., Yúfera M., Dinis M. T., and Aragão C., 2010: Dietary taurine supplementation enhances metamorphosis and growth potential of *Solea senegalensis* larvae. *Aquacult.*, **309**, 159-164.

Rust M. B., Barrows F. T., Hardy R. W., Lazur A. L., Naughten K., and Silverstein J., 2011: NOAA/USDA Alternative Feeds Initiative. The Future of Aquafeeds. NOAA Tech. Memo. NMFS-F/SPO-124 103 p.

Salze G., McLean E., Battle P. R., Schwarz M. H., and Craig S. R., 2010: Use of soy protein concentrate and novel ingredients in the total elimination of fish meal and fish oil in diets for juvenile cobia, *Rachycentron canadum*. *Aquacult.*, **298**, 294-299.

Schuller Levis G. B., and Park E., 2003: Taurine: new implications for an old amino acid. *FEMS Microbiol. Lett.*, **226**, 195-202.

Thiessen D. L., Maenz D. D., Newkirk R. W., Classen H. L., and Drew M. D., 2004: Replacement of fishmeal by canola protein concentrate in diets fed to rainbow trout (*Oncorhynchus mykiss*). *Aquac. Nutr.*, **10**, 379-388.

Trushenski J., Schwarz M., Bergman A., Rombenso A., and Delbos B., 2012: DHA is essential, EPA appears largely expendable, in meeting the n-3 long-chain polyunsaturated fatty acid requirements of juvenile cobia *Rachycentron canadum*. *Aquacult.*, **326-329**, 81-89.

Wacyk J., Powell M., Rodnick K., Overturf K., Hill R. A., and Hardy R., 2012: Dietary protein source significantly alters growth performance, plasma variables and hepatic gene expression in rainbow trout (*Oncorhynchus mykiss*) fed amino acid balanced diets. *Aquacult.*, **356-357**, 223-234.

Watson A. M., Barrows F. T., and Place A. R., 2014: Effects of graded taurine levels on juvenile cobia, *Rachychentron canadum*. *Nor. Am. J. Aquacult.*, **76**, 190-200.

Watson A. M., Barrows F. T., and Place A. R., 2013a: Taurine supplementation of plant derived protein and n-3 fatty acids are critical for optimal growth and development of cobia, *Rachycentron canadum*. *Lipids*, **48:9**, 899-913.

Watson A. M., Barrows F. T., and Place A. R., 2013b: Taurine supplemented plant protein based diets with alternative lipid sources for juvenile gilthead sea bream *Sparus aurata*. *J. Fish. Aquac.*, **4**, 59-66.

Watson A. M., Kissil G. W., Barrows F. T., and Place A. R., 2012: Developing a plant-based diet for cobia *Rachycentron canadum*. *Int. Aquafeed*, **15**, 34-38.

Xie S., He X., and Yang Y., 1998: Effects on growth and feed utilization of Chinese longsnout catfish *Leiocassis longirostris* Günther of replacement of dietary fishmeal by soybean cake. *Aquac. Nutr.*, **4**, 187-192.

水研センター研報，第40号，61－65，平成27年
Bull. Fish. Res. Agen. No. 40, 61－65, 2015

Availability of Fisheries By-Product Materials with Cadmium Removal Treatment as a Feed Ingredient for Fingerling Black Rockfish *Sebastes schlegeli*

Nobukazu SATOH[*1], Motoomi WAKASUGI[*2], and Shigeharu NOBUTA[*3]

Abstract: Squid liver and scallop-mid gut glands, which are generated and discarded as waste from the Japanese common squid and Japanese scallop, are rich in amino acids and lipids. However, the squid liver and scallop mid-gut glands contain cadmium (Cd) which is generally known as a harmful heavy metal for human health. Hirama *et al.*[*4] recently studied a technique for removal of Cd from squid viscera and scallop mid-gut glands and confirmed the efficacy of the acid leaching and electrolysis method to produce the fisheries by-product materials (meal and extract) with Cd removal treatment as a feed ingredient for cultured fish.

So far we have evaluated the nutritional value of squid viscera meal with Cd removal treatment (dCSVM), which contained 1.5–2.0 mg/kg Cd, as an alternative protein source to sardine meal in diets for fingerling black rockfish *Sebastes schlegeli*. In our study, it was clearly demonstrated that dCSVM with good protein digestibility could be substituted for 60% of sardine meal in diets for fingerling black rockfish without growth retardation, poor palatability and the problem of Cd accumulation. We suggested that dCSVM was superior to commercial squid viscera meal without Cd removal treatment (CSVM) as a high-quality feed ingredient for black rockfish based on the heavy metal accumulation in fish tissues.

Recently, we are investigating availability of mid-gut glands extract with Cd removal treatment (dCSMGE) as a feed ingredient for fingerling black rockfish. In the recent study, we found that weight gain, specific growth rate, and feed efficiency of fish fed the diet containing dCSMGE at 2% were significantly higher than those of the control. Furthermore, we confirmed that test diets containing dCSMGE was safe in terms of accumulation of Cd.

These results demonstrate that proper inclusion of dCSVM and dCSMGE is effective for the improvement of feed quality in practical diets for fingerling black rockfish.

Key words: squid viscera, scallop mid-gut glands extract, cadmium, alternative ingredients for fish meal with Cd removal treatment, black rockfish

The increasing feed cost in aquaculture production due to the increasing price of fish meal has been a serious problem recently. Therefore, the importance of studies on alternative protein sources for fish meal, a major ingredient for aquaculture feed, in diets has been increasingly realized (Gatlin *et al.*, 2007). However, it is widely recognized that high inclusion of alternative protein source in diets may result in poor palatability, particularly at low temperatures.

2015年1月30日受理 (Received on January 30, 2015)
*1 Fisheries Research Department Mariculture Fisheries Research Institute, Hokkaido Research Organization, Muroran, Hokkaido 051-0013, Japan
E-mail: sato-nobukazu@hro.or.jp
*2 IndustrialResearch Institute, Hokkaido Research Organization, Sapporo, Hokkaido 060-0819, Japan
*3 Fisheries Research Department Kushiro Fisheries Research Institute, Hokkaido Research Organization, Kushiro, Hokkaido 085-0027, Japan
*4 Hirama M., WakasugiM., TomitaK., KamadaT., TakahashiT., NobutaS., AsoS., SatohN., and IshidaR., 2013: Studies on the technique for removal of Cadmium from scallop mid-gut glands to produce fisheries by-product materials with Cadmium removal treatment as a feed ingredient for fish farming (in Japanese). Abstracts of the Annual Meeting of the Japanese Society of Fisheries Science. Japanese Society of Fisheries Science, Mie, Japan, September, p.77.

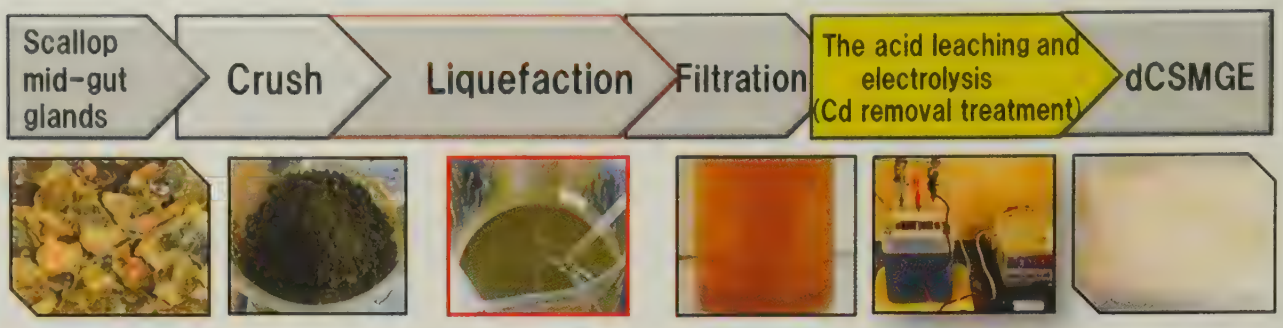


Fig. 1. Original production method of scallop mid-gut glands exract with Cd removal treatment (dCSMGE).

Squid liver, squid viscera and scallop mid-gut glands, which are generated and discarded as waste from the Japanese common squid *Todarodes pacificus* and Japanese scallop *Mizuhopecten yessoensis*, are rich in amino acids and lipids (Sakuta and Shimakage, 2004). Particularly, by-products of squid processing are important feed ingredients in aquaculture shrimps diets, since the diets contain squid by-products are effective for the improvement of growth performances and feed utilization in prawn species (Hertramp and Piedad-Pascual, 2000). In Hokkaido, Japan, these fisheries wastes are generated and wasted every year, and the volumes of squid viscera and scallop mid-gut glands were 9,324 and 30,533 metric ton in the 2010 fiscal year.

However, the squid liver and scallop mid-gut glands contain cadmium (Cd) at 19 and 39 mg per kg wet weight on average, respectively (Wakasugi *et al.*, 2005; Kruzynski, 2004). Cd is generally known as a harmful heavy metal for human health (Mai *et al.*, 2006). Wakasugi *et al.* (2009) recentlystudied a technique (Fig. 1) for removal of Cd from squid viscera and scallop mid-gut glands[*5] and confirmed the efficacy of acid leaching and electrolysis to produce fisheries by product materials (meal and extract) with Cd removal.This material was evaluated as a feed ingredient for cultured fish (Wakasugi, 2009). Consequently, they have developed squid viscera mealand scallop mid-gut glands exract with Cd removal treatment, dCSVM and dCSMGE, respectively.

Black rockfish, *Sebastes schlegeli is* an important species in Japan (Nakagawa *et al.*, 2007) and Korea (Bai, 2009) that is cultured for stock enhancement and aquaculture. However, information on the alternative protein sources of fish meal in diets for aquaculture is still limited. Aso *et al.* (1999) reported that scallop viscera meal with Cd removal treatment could substitute 10 % of brown fish meal in the diets for fingerling black rockfish.Lim *et al.* (2004) showed that dehulled soybean meal could replace fish meal up to 20% withoutsupplementation of methionine and lysine and 30% with these amino acids being supplemented in fingerling and growing black rockfish. Therefore, we studied availability of dCSVM and dCSMGE as a feed ingredient for fingerling black rockfish.The present article reviews information on the feed values of dCSVM and dCSMGE for black rockfish.

Availability of squid viscera as a feed ingredient for cultured-fish

Availability of various by-products in fisheries as a feed ingredient for aquaculture fish species was studied recentlyby many researchers, because by-products fromfisheries hold promise as potential protein substitutes for fish meal (Li *et al.*, 2004).

It has been reported that extrudeddiets containing 20–30 % raw squid liver could be used for rainbow trout *Oncorhynchus mykiss*, yellowtail *Seriola quinqueradiata*, and red seabream *Pagrus major* (Mastuda *et al.*, 2001a, b). Feeding studies have shown that the commercial squid viscera meal

[*5] Hirama M., WakasugiM., TomitaK., KamadaT., TakahashiT., NobutaS., AsoS., SatohN., and IshidaR., 2013: Studies on the technique for removal of Cadmium from scallop mid-gut glands to produce fisheries by-product materials with Cadmium removal treatment as a feed ingredient for fish farming (in Japanese). Abstracts of the Annual Meeting of the Japanese Society of Fisheries Science. Japanese Society of Fisheries Science. Mie, Japan, September, p.77.

without Cd removal treatment (CSVM) can be used as an alternative protein source in diets for various fish species; In fingerling black rockfish, Satoh *et al.* (2006) showed that CSVM could be substituted for 30% of sardine meal in a diet when growth performance was used as the dependent variable, while substitution of only 10% of sardine meal could be recommendedin terms of the heavy metal accumulation in the liver. Mai *et al.* (2006) evaluated the effects of dietary CSVM on the growth and Cd accumulation in tissues of Japanese seabass, *Lateolabrax japonicus*. They demonstrated that growth rates in fish fed diets contained 50 and 100g CSVM per kg diet were significantly higher than that in control fish, and that Japanese seabass did not accumulate Cd in the muscle (edible portion), although Cd concentrations in fish kidneys, livers and gills were at high levels (0.4–5.9 mg per kg dry weight). In the paper, they also pronounced that a long-term feeding trial was necessary to investigate Cd accumulation in cultured Japanese seabass. More recently, Wang *et al.* (2012) reviewed that several fish diets supplemented with CSVM contained high Cd levels, 51–116 mg/kg diet.

Availability of dCSVM as a feed ingredient for fingerling black rockfish

We have evaluated the nutritional value of dCSVM, which contained 1.5–2.0 mg/kg Cd, as an alternative protein source to sardine meal in diets for fingerling black rockfish (Satoh *et al.*, 2013a). The Cd level of dCSVM was less than 3.0 mg/kg, which is the allowable level of Cd in livestock feed ingredients according to the Law Concerning Safety Assurance and Quality Improvement of Feeds in Japan. In this study, it was demonstrated that dCSVMwith good protein digestibility could be substituted for 60% of sardine meal in diets for juvenile black rockfish without growth retardation, poor palatability and the Cd accumulation, although feed efficiency gradually decreased with the increase of dCSVM inclusion. In that paper, we suggested that dCSVM was superior to CSVM as a high-quality feed ingredient for black rockfish based on the heavy metal accumulation in fish tissues. Furthermore, we found that appropriate replacement (30–60%) of sardine meal by dCSVM was effective for stimulation of feeding in fingerling black rockfish, and that supplementation of methionine and lysine was not necessary with the inclusion of dCSVM. From these results, we concluded that dCSVMwas safe and useful as an alternative protein source in fingerling black rockfish diets.

Availability of dCSMGE as a feed ingredient for fingerling black rockfish

We also investigated availability of dCSMGE, which contains about 0.5 mg/kg Cd, as a feed ingredient for fingerling black rockfish[*4]. The total free amino acids content (mg/kg dry matter) of dCSMGE is about 30,000, while that of fish meal is about 800. dCSMGE contain amino acids effective for feeding stimulants and growth enhancement in various fish species (Mackie and Mitchell, 1985, Shimizu *et al.*, 1999, Ikeda *et al.*, 2012). In the recent feeding trial for 65 days, we found that weight gain, specific growth rate, and feed efficiency of fish fed the diet containing dCSMGE at 2% of the diet were significantly higher than those of the control[*6]. Moreover, we confirmed that test diets containing dCSMGE was safe in terms of accumulation of Cd[*6].

Kumai *et al.* (1989) found that commercial scallop extract as a flavor in the diet for ocelate puffer *Takifugu rubripes* promoted feed intake and digestive and absorptive functions in the ocelate puffer. Kikuchi and Furuta (2009) reported that blue mussel extract in diets based on fish and soybean meals for tiger puffer would be an effective feeding stimulant. We observed that dCSMGE improved feeding activity of barfin flounder *Veraspermoseri* in the short-term feeding experiment at low temperatures[2*7]. dCSMGE may be also an effective

*6 Satoh N., IshidaR., NobutaS., AsoS., WakasugiM., TomitaK., HiramaM., and TakahashiT., 2013: Availability of scallop mid-guts extract with cadmium removal treatment as a feed ingredient for fingerling black rockfish (in Japanese). Abstracts of the Annual Meeting of the Japanese Society of Fisheries Science.Japanese Society of Fisheries Science, Mie, Japan, September, p.42.

*7 Satoh N., IshidaR., NobutaS., AsoS., WakasugiM., TomitaK., HiramaM., and TakahashiT., 2013: Availability of scallop mid-guts extract with cadmium removal treatment as a feed ingredient for juvenile barfin flounder (in Japanese). Abstracts of the Annual Meeting of the Japanese Society of Fisheries Science. Japanese Society of Fisheries Science, Tokyo, Japan, March, p.135.

feed ingredient for the improvement of growth performance, feed utilization, and feeding activity in fish.

Krill extract and meal are often added to aquaculture feed to improve of platability (Shimizu, 1999) and digestibility (Satoh, 2003), but the supplementation in the diets is mostly limited for larval and juvenile fish because of the high price. To date, we estimate the cost of producing dCSMGE (semi-liquid form) will be below half of cost of krill ingredients. On the other hand, the producing cost of dCSMGE in powder form is higher than that in semi-liquid form, since producing of dCSMGE powder requires energy to dry. Interestingly, Kader *et al.* (2012) found that fermented soybean meal and squid by-product blend (1:1) could be substituted for about 40% of fish meal protein in the diet of Japanese flounder. Likewise, fermented soybean meal and scallop by-product blend (1:1) was able to replace at least 30% fish meal protein in red seabream diet (Kader *et al.*, 2011). These blends are effective for producing low-cost and healthy aquaculture feed, and the fermentation technique may be an effective measure of producing dCSMGE powder.

Conclusion

Our results demonstrate that proper inclusion of dCSVM and dCSMGE is effective for the improvement of feed quality in practical diets for fingerling black rockfish. Future research is needed to clarify the nutritional value of dCSVM and dCSMGE as a feed ingredient for aquaculture fish species other than black rockfish.

Acknowledgements

The research was financially supported by a Research Project for Utilizing Advanced Technologies in Agriculture, Forestry, and Fisheries (grant no. 18031), Ministry of Agriculture, Forestry, and Fisheries, Government of Japan and partly by Hokkaido Government.

References

Aso S., Kitagawa M., Saitoh S., and Mori T., 1999: Utilization of scallop visceral meal in the diets for fingerling rockfish, *Sebastesschlegeli* (in Japnanese with English abstract). *Sci. Rep. Hokkaido Fish. Exp. Stn.*, **56**, 143-147.

Bai S. C., and Okorie O. E., 2009: Korean rockfish production in Korea. *Global aquaculture advocate*, Jan./Feb, 41-42.

Gatlin Ⅲ D. M., Barrows F. T., Brown P., Dabrowski K., Gaylord G., Hardy R. W., Herman E., Hu G., Krogdahl A., Nelson R., Overturf K., Rust M., Sealey W., Skonberg D., Souza E. J., Stone D., Wilson R., and Wurtele E., 2007: Expanding the utilization of sustainable plant products inaquafeeds: a review. *Aquaclt. Res.*, **38**, 551-579.

Hertramph J. W., and Piedad Pacual F., 2000: Handbook on ingredients for aquaculture feeds. 1st ed., Kluwer Academic, Dordrecht, p.403-410.

Ikeda I., Okamoto Y., and Oda K., 2012: Identification of feeding stimulants for Japanese flounder in muscle extract of Jack mackerel. *Aquaclt. Sci.*, **60**, 195-198.

Kader M. A., Koshio S., Ishikawa M., Yokoyma S., Bulbul M., Honda Y., Mamauag R. E., and Laining A., 2010: Growth, nutrient utilization, oxidative condition, and element composition of juvenile red sea bream *Pagrus major* fed with fermented soybean meal and scallop by-product blend as fish meal replacement. *Fish. Sci.*, **77**, 119-128.

Kader M. A., Koshio S., Ishikawa M., Yokoyma S., Bulbul M., Nguyen B. T., GaoJ., and Laining A., 2012: Can fermented soybean meal and squid by-product blend be used as fishmeal replacements for Japanese flounder (*Paralichtysoliverceus*)? *Aquaclt. Res.*, **43**, 1427-1438.

Kikuchi K., and Furuta T., 2009: Inclusion of blue mussel extract in diets based on fish and soybean meals for tiger puffer *Takifugurubripes*. *Fish. Sci.*, **75**, 183-189.

Kumai H., Kimura I., Nakamura M., Takii K., and Ishida H., Studies on digestive system and assimilation of a flavored diet in Ocellate puffer. *Nippon Suisan Gakkaishi*, **55**, 1035-1043.

Kruzynski G. M., 2004: Cadmium in oysters and scallops: the BC experience. *Toxic. Letters* **148**, 159-169.

Li P., Wang X., Hardy R. W., and Gatrin Ⅲ D. M., 2004: Nutritional value of fisheries by-catch and by-product meals in the diet of red drum (*Sciaenopsocellatus*). *Aquaculture*, **236**, 485-496.

Lim S. R., Choi S. M., Wang X. J., Kim K. W., Shin I. S., Min T. S., and Bai S. C., 2004: Effects of dehulled soybean meal as a fish meal replacer in diets for fingerling and growing Korean rockfish *Sebastes schlegeli*. *Aquaculture*, **231**, 457-468.

Mackie A. M., and Mitchell A. I., 1985: Identification of gustatory feeding stimulants for fish-applications in aquaculture, *In* C.B. Cowey, A. M. Mackie, and J. G. Bell (eds.), Nutrition and Feeding in Fish, Academic Press, London, p.177-189.

Mai K., Li H., Ai Q., Duan Q., Xu W., Zhang C., Zang L., Tan B., and Liufu Z., 2006: Effects of dietary squid viscera meal on growth and cadmium accumulation in tissues of Japanese seabass, *Lateolabrax japonicus* (Cuiver 1828). *Aquac. Res.*, **37**, 1063-1069.

Matsuda Y., Hayashi A., Yoshitomi B., Takeuchi T., 2001: Preliminary studies on effect of the supplementation of raw squid liver to extruded pellet on the digestibility of dietary nutrients and the feed performance for raibow trout (in Japanese with English abstract). *Aquaclt. Sci.*, **49**, 377-381.

Matsuda Y., Hayashi A., Yoshitomi B., Kanazawa A., and Takeuchi T., 2001: Effect of the combination of raw squid liver to extruded pellet on growth performance and digestibility of dietary nutrients for Yelowtail and Red seabream (in Japanese with English abstract). *Aquaclt. Sci.*, **49**, 483-488.

Nakagawa M., Okouchi H., AdachiJ., Hattori K., and Yamashita Y., 2007: Effectiveness of stock enhancement of hatchery-reared black rockfish Sebastesschlegeli in Yamada Bay-Evaluation by a Fish Market survey. *Aquaculture*, **263**, 295-302.

Sakuta Y., and Shimakage K., 2004: Development of recycling technology for scallop waste by hydrometallurgical process (in Japanese with English abstract). *Shigen-to-Sozai*, **120**, 71-77.

Satoh K., 2003: Effects of supplement with krill extract and krill meal to diet on the growth performance and protein digestibility of yellowtail during low water temperature (in Japanese with English abstract). *Aquaclt. Sci.*, **51**, 93-99.

Satoh N., Fujioka T., and Nobuta S., 2006: Availability of commercial squid liver meal as diets of fingerling rockfish (in Japanese with English abstract). *Nippon Suisan Gakkaishi*, **72**, 401-407.

Satoh N., Nobuta S., Wakasugi M., Satoh S., and Takeuchi T., 2013: Availability of squid viscera meal with cadmium removal treatment as a feed ingredient for fingerling black rockfish *Sebastes schlegeli.Fish. Sci.*, **79**, 259-267.

Shimizu C., Allahpichay I., Tokoro T., Shirakawa Y., 1990: Feeding stimulation in sea bream, *Pagrus major*, fed diets supplementedwith Antarctic krill meals. *Aquaculture*, **89**, 43-53.

Wang W. X., Onsaint S., and Dang F., 2012: Dietary bioavailability of cadmium, inorganic mercury, and zinc to a marine fish: Effects of food composition and type. *Aquaculture*, **356-357**, 98-104.

Wakasugi M., Tomita K., Takahashi T., Nagano N., and Sakuta Y., 2005: Development of processing and utilizing technology of squid viscera (Part Ⅳ) (in Japnanese with English abstract). Sci. Rep. *Hokkaido Industrial Res. Inst.*, **306**, 41-51.

Wakasugi M., 2009. Development of high quality diet using squid viscera for fish farming (in Japanese). *Aquaclt. Mag.*, **574**, 28-31.

水研センター研報，第40号，67－68，平成27年
Bull. Fish. Res. Agen. No. 40, 67－68, 2015

Cholecystokinin and Trypsin Responses of Larval Red Drum (*Sciaenops ocellatus*) in Response to Algae, Live Prey, and Inert Particles

Ken WEBB*1

Abstract: In an attempt to better understand the problems in weaning larval fish to artificial diets, our lab has begun to investigate the role of the digestive hormone cholecystokinin (CCK). While there are a number of other labs also investigating CCK and other digestive hormones such as bombesin, PPY, and gastrin; research into the roles of these hormones in fish is still in its infancy. Previous research with red drum larvae suggests that some component of rotifers and algae enable red drum larvae to more efficiently utilize microparticulate diets than when these are not included in the culture system. The current work investigated the impact of soluble components of rotifers and algae on the CCK and trypsin responses of larval red drum at 6 and 10 days post hatch (DPH) as well as the response of red drum larvae to ingestion of inert polystyrene particles at 10 DPH. Introduction of homogenized rotifers was shown to significantly increase whole body CCK levels, CCK mRNA, and trypsin activity in 6 DPH red drum larvae, but not in 10 DPH larvae. Homogenates of *Isochrysis galbana* did not significantly affect CCK or trypsin at either age. Ingestion of the polystyrene particles was increased in response to the presence of rotifer homogenate and both CCK mRNA and trypsin activity was increased as well. This research suggests that there is a soluble component of rotifers that can upregulate digestive function in larval red drum, at least in 6 DPH larvae, as well as influence consumption.

Annotated Bibliography

Dockray G. J., 2012: Cholecystokinin. Current Opinion in Endocrinology, Diabetes & Obesity, **19(1)**, pp 8–12.

The author provides an excellent review of the current understanding of the role of Cholecystokinin (CCK) in satiety and to a lesser extent, digestion. The author focuses on the role of CCK in the cephalic phase and reviews current knowledge of both CCK activators and targets. The author also discussed the role of Leptin in potentiating the effect on CCK on vagal afferent neurons. Of particular note in this manuscript, the author mentions the role of GPR40 and long-chain fatty acids in the secretion of fatty acids while much of the other literature focuses on protein hydrolysate / amino acid roles in promoting CCK secretion.

Liou A. P., Chavez D. I., Espero E., Hao S., Wank S. A., and Raybould H. E., 2010: Protein hydrolysate-induced cholecystokinin secretion from enteroendocrine cells is indirectly mediated by the intestinal oligopeptide transporter $PepT_1$. *Am. J. Physiol. Gastrointest. Liver Phsyiol.*, **300: G**, G895-G902.

The authors investigate the role of the $PepT_1$ transporter as the direct mediator of Cholecystokinin (CCK) secretion in response to protein hydrolysate. Before this work, $PepT_1$ was considered a likely mediator of direct mediation of CCK secretion due to previous work that showed a synthetic dipeptide, Gly-Sar, used in $PepT_1$ kinetic studies caused a dose-dependent inhibition of gastric motility consonant with CCK secretion. The work demonstrated that while Gly-Sar did inhibit gastric motility it had no effect on eliciting CCK secretion from CCK-eGFP

2015年1月30日受理（Received on January 30, 2015）
*1 Environmental and Fisheries Sciences, National Marine Fisheries Service, Northwest Fisheries Science Center, 2725 Montlake Boulevard East, Seattle, WA 98112
E-mail: Ken.Webb@NOAA.gov

cells. Based on this work, the authors concluded that protein detection by intestinal I cells likely includes both direct and indirect detection but that $PepT_1$ does not have a direct role in CCK secretion. The authors do however suggest that $PepT_1$ may function indirectly by stimulating a diazepam-binding inhibitor.

Cudenneca B., Fouchereau-Peron M., Ferry F., Duclos E., and Ravallec R., 2012: *In vitro* and *in vivo* evidence for a satiating effect of fish protein hydrolysate obtained from blue whiting (*Micromesistius poutassou*) muscle. *J. Func. Foods.*, **4**, 271–277.

The authors in this work examine the role of protein hydrolysate from blue whiting on CCK secretion in STC-1 cells as well as on long and short-term food intake of rats fed blue whiting muscle hydrolysate (BWMH). The authors found that in the *in vitro* studies on STC-1 cells, peptides from the BWMH stimulated CCK secretion in a dose-dependent response similar to that seen from other types of protein hydrolysate. In the *in vivo* study, the authors found that BWMH produced short-term reductions in food intake but that this reduction was not reflected in the long term. The *in vivo* study did show that blood CCK and GLP-1 levels were more than doubled in rats fed 100 and 250mg of BWMH following a 24h fast lending credence that these may be involved in the short-term decrease in consumption.

水研センター研報，第40号，69－74，平成27年
Bull. Fish. Res. Agen. No. 40, 69－74, 2015

Effect of Feed Ingredients on Digestive Enzyme Secretion in Fish

Koji MURASHITA[*1], Haruhisa FUKADA[*2], Noriyuki TAKAHASHI[*2], Noriko HOSOMI[*2], Hiroyuki MATSUNARI[*1], Hirofumi FURUITA[*1], Hiromi OKU[*1], and Takeshi YAMAMOTO[*1]

Abstract: In response to the limitation in the global supply of fish meal, the traditional protein source used in aquaculture feed, efforts have increasingly been focused on the use of alternative protein sources of plant origin. However, plant ingredients may cause growth retardation in aquaculture fish species. Feed nutrients must be digested for their utilization, and so pancreatic digestive enzymes have essential roles for the digestion. Also, cholecystokinin (Cck) is known to be a hormone that stimulates the secretion of digestive enzymes in vertebrates. To improve the utilization of plant based diets in fish, we investigated the effects of various feed ingredients on secretion of digestive enzymes in red seabream *Pagrus major* and yellowtail *Seriola quinqueradiata*, which are commercially important aquaculture species in Japan.

We first investigated the effect of soybean meal on digestive enzyme secretion in red seabream. Activities of pancreatic digestive enzymes (trypsin, chymotrypsin, lipase, amylase) in the intestinal content of a soybean meal based diet (SBM) fed fish were lower than those of a fish meal based diet (FM) fed fish. Also, lower gene expression levels of the digestive enzymes in the hepatopancreas were observed in the SBM fed fish compared with the FM fed fish. These data indicate the FM diet stimulated the secretion/synthesis of pancreatic digestive enzymes to a greater degree than the SBM diet. Then, we tried to identify the stimulation factor in fish meal. Administration of a FM water-soluble fraction increased the gene expression of *trypsin, lipase, cck and cck receptor* (*cck-1r*) in yellowtail, suggesting the enzyme stimulation factor may exist in the water-soluble fraction of FM. Supplementation of the enzyme stimulation factor, although not identified yet, may improve the utilization of plant based diets in aquaculture fish species.

Key words: red seabream (*Pagrus major*), yellowtail (*Seriola quinqueradiata*), pancreatic digestive enzymes, fish meal, soybean meal

Fish meal (FM), which is primarily produced from small pelagic fish such as jack mackerel and anchovy, has long been used as the major protein ingredient for fish feeds in aquaculture. However, as the wild stocks of these pelagic fish species become increasingly limited, development of alternative feeds is considered essential to the expansion of sustainable aquaculture worldwide. In response to such a limitation in the global supply of FM, efforts have increasingly been focused on the use of alternative protein sources of plant origin. Especially, soybean meal (SBM) is known to be the most common fish meal-replacement due to its price, high availability in the market and relatively well-balanced amino acid profile. However, fish fed with diets containing high concentrations of plant protein often show low growth performance and digestibility of feed (Gaylord *et al.*, 2008; Glencross *et al.*, 2007).

Feed nutrients must be digested for their utilization, and pancreatic digestive enzymes have essential roles for the digestion; trypsin and chymotrypsin are the main pancreatic proteases, lipase is the major pancreatic lipolytic enzyme, and amylase is known as the major pancreatic digestive

2015年1月30日受理（Received on January 30, 2015）
*1 National Research Institute of Aquaculture, Fisheries Research Agency, Tamaki, Mie 519-0423, Japan
E-mail: kojim at affrc.go.jp
*2 Faculty of Agriculture, Kochi University, 200 Monobe, Nankoku, Kochi 783-8502, Japan

enzyme for carbohydrates. The exocrine pancreatic enzymes secretion is controlled by both neuronal and hormonal factors (Konturek *et al.*, 2003). In most vertebrates, a peptide hormone cholecystokinin (Cck) is known to be one of the key physiological regulators of pancreatic secretion (Einarsson *et al.*, 1997; Kofuji *et al.*, 2007). Recently, the *cck* gene has been identified in several fish species such as Atlantic herring *Clupea harengus* (Kamisaka *et al.*, 2005), Atlantic salmon *Salmo salar* (Murashita *et al.*, 2009), yellowtail *Seriola quinqueradiata* (Murashita *et al.*, 2006) and red drum *Sciaenops ocellatus* (Webb *et al.*, 2010), and is mainly expressed in the anterior part of the intestine (including the pyloric caeca) (Murashita *et al.*, 2006). Further, the *cck receptor* (*cck-1r*) gene has also been identified in yellowtail and is principally expressed in the pyloric caeca (Furutani *et al.*, 2013).

To improve the utilization of plant based diets in fish, we investigated the effects of various feed ingredients on secretion of digestive enzymes in red seabream *Pagrus major* and yellowtail, which are commercially important aquaculture species in Japan.

Materials and Methods

Effect of soybean meal on digestive enzymes secretion in red seabream: Juvenile red seabream were used for this experiment. Isonitrogenous and isolipidic FM based and SBM based diets were prepared (diet FM and diet SBM). The fish were fed the experimental diets for 6 weeks, and the hepatopancreas and intestinal content of the fish were sampled (n = 6). Activities of pancreatic digestive enzymes (trypsin, chymotrypsin, lipase and amylase) in the intestinal content (n = 7) were analyzed according to Murashita *et al.* (2007). Gene expression levels of the enzymes in the hepatopancreas were evaluated by real-time quantitative PCR (qPCR) using the specific primers for red seabream digestive enzymes.

Effect of fish meal on gene expression levels of *trypsin*, *lipase*, *cck* and *cck-1r* in yellowtail: Water-soluble and water-insoluble fractions of FM were prepared. The same amount of the two FM fractions on crude protein basis were orally administrated to juvenile yellowtail according to Murashita *et al.* (2008). The pyloric caeca was sampled from the fish,

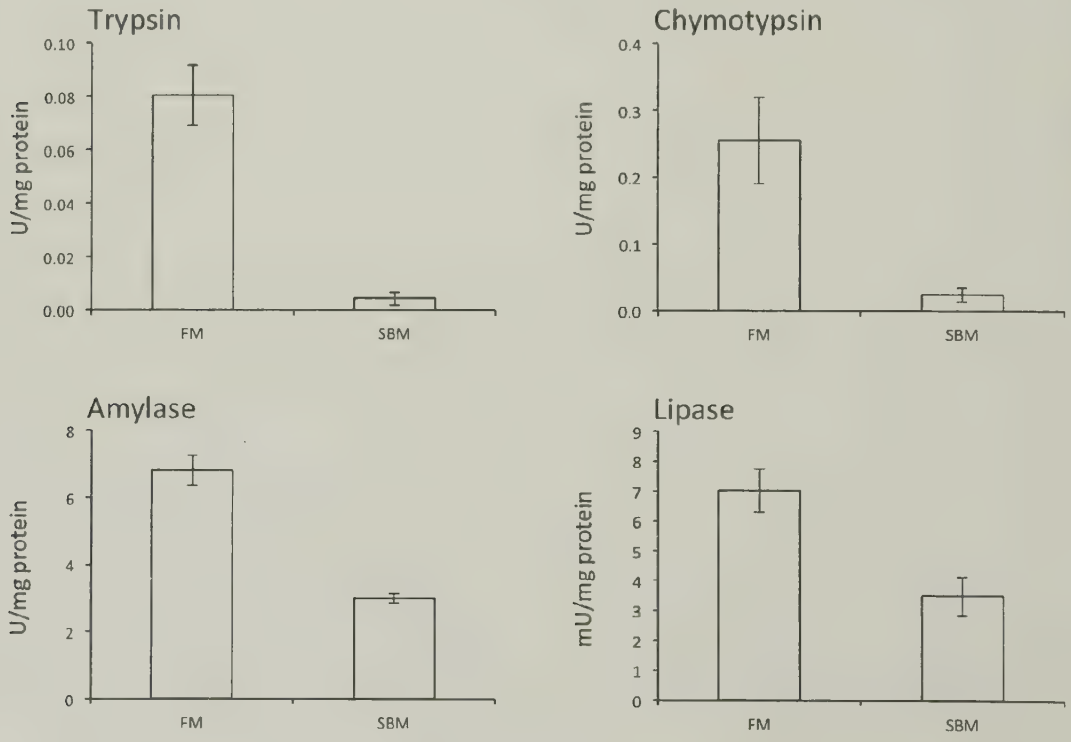


Fig. 1. Effects of fish meal (FM) and soybean meal (SBM) based diets on activities of the pancreatic digestive enzymes in intestinal content of red seabream. Values are mean ± SE (n = 7 fish).

and the gene expression levels of *trypsin, lipase, cck* and *cck-1r* were analyzed by qPCR according to Furutani *et al.* (2013).

Results and Discussion

Effect of soybean meal on digestive enzymes secretion in red seabream: Activities of all four pancreatic digestive enzymes examined in the intestinal content of SBM fed fish were lower than those of FM fed fish (Fig. 1), indicating the amounts of enzymes secreted into the intestine in the SBM fed fish were lower than those of the FM fed fish. Also, lower gene expression levels of the digestive enzymes in the hepatopancreas were observed in the SBM fed fish compared with the FM fed fish (Fig. 2), which is in line with the report in yellowtail; orally administrated FM increased the *trypsin* and *lipase* gene expressions in the pyloric caeca, but not in fish administrated SBM (Furutani *et al.*, 2012). These data indicate that SBM does not fully stimulate the secretion/synthesis of the pancreatic digestive enzymes, or, FM strongly stimulates the digestive enzymes secretion/synthesis.

Effect of fish meal on gene expression levels of *trypsin*, *lipase*, *cck* and *cck-1r* in yellowtail: Administration of a FM water-soluble fraction increased the gene expressions of *trypsin, lipase, cck* and *cck-1r* in yellowtail, whereas those of fish administrated a FM water-insoluble fraction did not (Fig. 3, brief results), suggesting the enzyme stimulation factor may exist in the water-soluble fraction of FM. We are trying to identify the enzyme stimulation factor from the FM water-soluble fraction.

The main component of the FM water-soluble fraction may be very small peptides and/or free amino acids (data not shown). It is known that some amino acids strongly stimulate CCK release in rodents (Daly *et al.*, 2013); such a mechanism might also exist in fish. Supplementation of the enzyme stimulation factor may improve the utilization of plant based diets in aquaculture fish species.

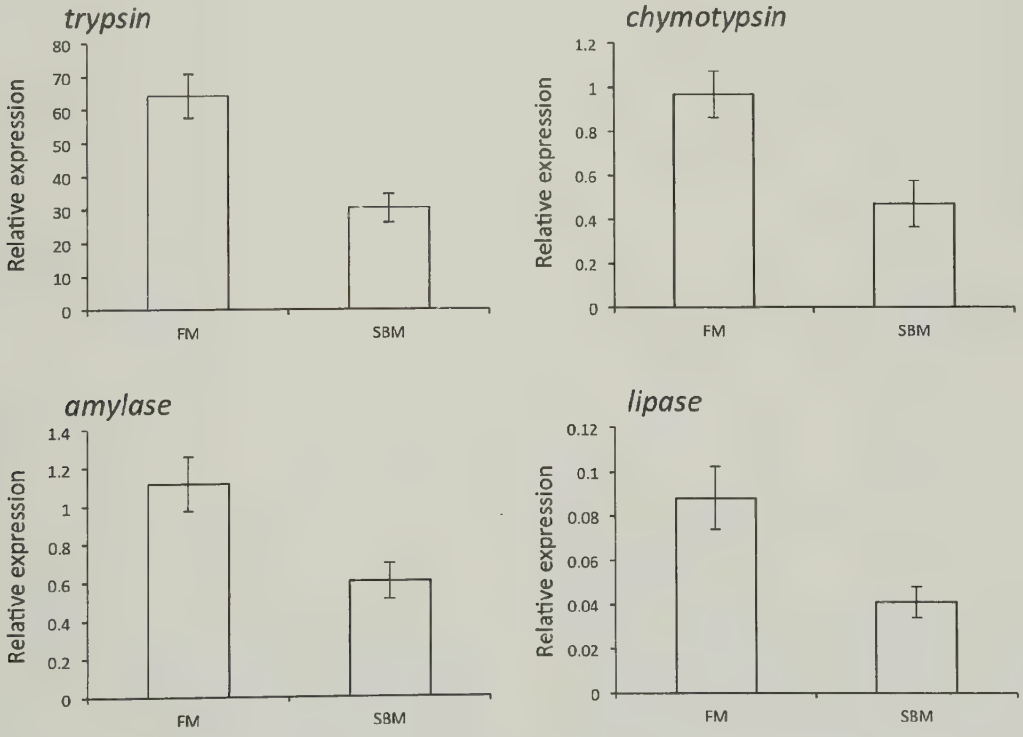


Fig. 2. Effects of fish meal (FM) and soybean meal (SBM) based diets on gene expression of pancreatic digestive enzymes in the hepatopancreas of red seabream. Data for each gene is represented as mean calculated copy number normalized against red seabream *ef1a* copy numbers. Values are mean ± SE (n = 6 fish).

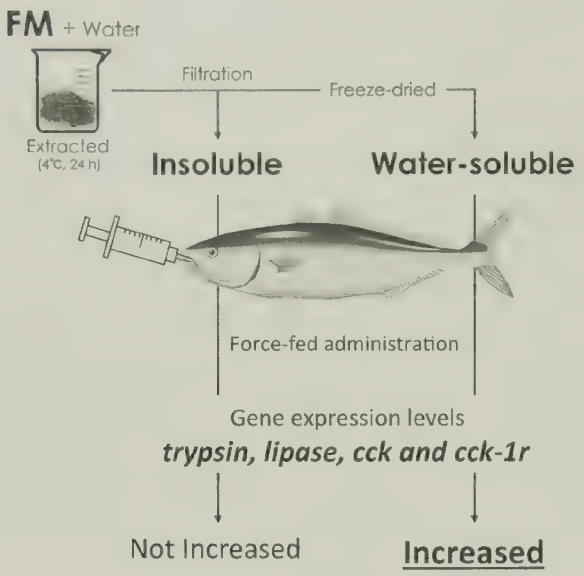


Fig. 3. Effect of fish meal on gene expression levels of *trypsin*, *lipase*, *cck* and *cck-1r* in yellowtail. The schematic diagram of the experiment is presented with brief results.

Acknowledgments

This work was partly supported by JSPS Grant-in-Aid for Scientific Research (B) (Grant Number: 24380117) to H. Fukada and K. Murashtia, and by the Fisheries Agency, Government of Japan.

References

Daly K., Al-Rammahi M., Moran A., Marcello M., Ninomiya Y., and Shirazi-Beechey S. P., 2013: Sensing of amino acids by the gut-expressed taste receptor T1R1-T1R3 stimulates CCK secretion. *Am. J. Physiol.*, **304**, G271-G282.

Einarsson S., Davies P. S., and Talbot C., 1997: Effect of exogenous cholecystokinin on the discharge of the gallbladder and the secretion of trypsin and chymotrypsin from the pancreas of the Atlantic salmon, *Salmo salar* L. *Comp. Biochem. Physiol. C*, **117**, 63-67.

Furutani T., Masumoto T., and Fukada H., 2012: Response of cholecystokinin and digestive enzyme mRNA levels to various feed ingredients in yellowtail *Seriola quinqueradiata*. *Fish. Sci.*, **78**, 1075-1082.

Furutani T., Masumoto T., and Fukada H., 2013: Molecular cloning and tissue distribution of cholecystokinin-1 receptor (CCK-1R) in yellowtail *Seriola quinqueradiata* and its response to feeding and in vitro CCK treatment. *Gen. Comp. Endocrinol.*, **186**, 1-8.

Gaylord T. G., Barrows F. T., and Rawles S. D., 2008: Apparent digestibility of gross nutrients from feedstuffs in extruded feeds for rainbow trout, *Oncorhynchus mykiss*. *J. World Aquacult. Soc.*, **39**, 827-834.

Glencross B. D., Booth M., and Allan G. L., 2007: A feed is only as good as its ingredients - a review of ingredient evaluation strategies for aquaculture feeds. *Aquacult. Nutr.*, **13**, 17-34.

Kamisaka Y., Drivenes Ø., Kurokawa T., Tagawa M., Rønnestad I., Tanaka M., and Helvik J. V., 2005: Cholecystokinin mRNA in Atlantic herring, *Clupea harengus*—molecular cloning, characterization, and distribution in the digestive tract during the early life stages. *Peptides*, **26**, 385-393.

Kofuji P. Y. M., Murashita K., Hosokawa H., and Masumoto T., 2007: Effects of exogenous cholecystokinin and gastrin on the secretion of trypsin and chymotrypsin from yellowtail (*Seriola quinqueradiata*) isolated pyloric caeca. *Comp. Biochem. Physiol. A*, **146**, 124-130.

Konturek S. J., Pepera J., Zabielski K., Konturek P. C., Pawlik T., Szlachcic A., and Hahn E. G., 2003: Brain-gut axis in pancreatic secretion and appetite control. *J. Physiol. Pharmacol.*, **54**, 293-317.

Murashita K., Fukada H., Hosokawa H., and Masumoto T., 2006: Cholecystokinin and peptide Y in yellowtail (*Seriola quinqueradiata*): Molecular cloning, real-time quantitative RT-PCR, and response to feeding and fasting. *Gen. Comp. Endocrinol.*, **145**, 287-297.

Murashita K., Fukada H., Hosokawa H., and Masumoto T., 2007: Changes in cholecystokinin and peptide Y gene expression with feeding in yellowtail (*Seriola quinqueradiata*): Relation to pancreatic exocrine regulation. *Comp. Biochem. Physiol. B*, **146**, 318-325.

Murashita K., Fukada H., Rønnestad I., Kurokawa T., and Masumoto T., 2008: Nutrient control

of release of pancreatic enzymes in yellowtail (*Seriola quinqueradiata*): Involvement of CCK and PY in the regulatory loop. *Comp. Biochem. Physiol. A*, **150**, 438-443.

Murashita K., Kurokawa T., Nilsen T. O., and Rønnestad I., 2009: Ghrelin, cholecystokinin, and peptide YY in Atlantic salmon (*Salmo salar*): molecular cloning and tissue expression. *Gen. Comp. Endocrinol.*, **160**, 223-235.

Webb K. A., Khan I. A., Nunez B. S., Rønnestad I., and Holt G. J., 2010: Cholecystokinin: Molecular cloning and immunohistochemical localization in the gastrointestinal tract of larval red drum, *Sciaenops ocellatus* (L.). *Gen. Comp. Endocrinol.*, **166**, 152-159.

Annotated Bibliography

Murashita K., Fukada H., Rønnestad I., Kurokawa T., and Masumoto T., 2008: Nutrient control of release of pancreatic enzymes in yellowtail (*Seriola quinqueradiata*): Involvement of CCK and PY in the regulatory loop. *Comp. Biochem. Physiol. A*, **150**, 438-443.

Cholecystokinin (Cck) and neuropeptide Y (Npy)-related peptides are the key regulators of pancreatic enzyme secretion in vertebrates. Cck stimulates enzyme secretion whereas peptide Y (Py or Pyyb), a Npy-related peptide, plays an antagonistic role to that of Cck. In fish, very little is known about the effects of different nutrients on the synthesis of Cck and Py in the digestive tract, and the mechanism by which Cck and Py actually regulate digestive enzyme secretion is not well understood. In order to determine stimulating effects of different nutrients on the synthesis of Cck and Py in yellowtail (*Seriola quinqueradiata*), *cck* and *py* mRNA levels in the digestive tract were measured after oral administration of a single bolus of either phosphate-buffered saline (PBS: control), starch (carbohydrate), casein (protein), oleic acid (fatty acid) or tri-olein (triglyceride). In addition, in order to confirm the synthesis and secretion of digestive enzymes, the mRNA levels and enzymatic activities of three digestive enzymes (*lipase*, *trypsin* and *amylase*) were also analyzed. Casein, oleic acid and tri-olein increased the synthesis of *lipase*, *trypsin* and *amylase*, while starch and PBS did not affect the activity of any of these enzymes. *cck* mRNA levels rose, while *py* mRNA levels were reduced in fish administered casein, oleic acid and tri-olein. These results suggest that in yellowtail, Cck and Py maintain antagonistic control of pancreatic enzyme secretion after intake of protein and/or fat.

Furutani T., Masumoto T., and Fukada H., 2012: Response of cholecystokinin and digestive enzyme mRNA levels to various feed ingredients in yellowtail *Seriola quinqueradiata*. *Fish. Sci.* **78**, 1075-1082.

Cholecystokinin (Cck) is the key regulator hormone that stimulates the secretion of digestive pancreatic enzymes in vertebrates. In fish, little is known about the mechanism of induction of Cck in the digestive tract by different feed ingredients. To investigate the response of *cck* and digestive enzymes to fish feed ingredients in yellowtail *Seriola quinqueradiata*, we performed a series of experiments in which we measured the mRNA levels of *cck*, *trypsin*, and *lipase* after oral administration of a single bolus of various ingredients. We administered fish meal and fish oil in experiment 1; high and low concentrations of fish meal in experiment 2; and five different dietary protein sources (fish meal, soybean meal, soy protein concentrate, corn gluten meal, and glutamic acid fermentation by-products) in experiment 3. In experiments 1 and 3, only fish meal significantly increased the mRNA level of *cck* and digestive enzyme. In experiment 2, a high concentration of fish meal [20 % (w/v)] significantly increased the *cck* and *trypsin* mRNA levels, but a low concentration of fish meal [1 % (w/v)] did not. These results suggest that high concentrations of fish meal (the protein source in fish feed) has the most potent effect on stimulation of *cck* synthesis and secretion of digestive enzymes in yellowtail.

Furutani T., Masumoto T., and Fukada H., 2013: Molecular cloning and tissue distribution of cholecystokinin-1 receptor (CCK-1R) in yellowtail *Seriola quinqueradiata* and its response to feeding and *in vitro* CCK treatment. *Gen. Comp. Endocrinol.* **186**, 1-8.

In vertebrates, the peptide cholecystokinin (Cck) is

one of the most important neuroregulatory digestive hormones. Cck acts via Cck receptors that are classified into two subtypes, Cck-1 receptor (Cck-1r; formally Cck-a) and Cck-2 receptor (formally Cck-b). In particular, the Cck-1r is involved in digestion and is regulated by Cck. However, very little information is known about Cck-1r in fish. Therefore, we performed molecular cloning of *cck-1r* cDNA from the digestive tract of yellowtail *Seriola quinqueradiata*. Phylogenetic tree analysis showed a high sequence identity between the cloned yellowtail *cck receptor* cDNA and *cck-1r*, which belongs to the *cck-1r* cluster. Furthermore, the expression of yellowtail *cck receptor* mRNA was observed in gallbladder, pyloric caeca, and intestines, similarly to *cck-1r* mRNA expression in mammals, suggesting that the cloned cDNA is the yellowtail *cck-1r*. In *in vivo* experiments, the *cck-1r* mRNA levels increased in the gallbladder and pyloric caeca after feeding, whereas *in vitro*, mRNA levels of *cck-1r* and digestive enzymes in cultured pyloric caeca increased by the addition of Cck. These results suggest that Cck-1r plays an important role in digestion stimulated by Cck in yellowtail.

水研センター研報，第40号，75－77，平成27年
Bull. Fish. Res. Agen. No. 40, 75－77, 2015

Development and Characterization of Several Open Formula Reference Diets for Marine Fish Larvae

Michael B. RUST*1, Frederic T. BARROWS*2, Mark DRAWBRIDGE*3, Emily R. HART*1, Kevin STUART*3, Ken WEBB*1, Harold J. BARNETT*1, Peter M. NICKLASON*1, and Ronald B. JOHNSON*1

Abstract: A limiting constraint in the development and growth of marine aquaculture is successful hatchery production. Presently, larval finfish are raised using live feeds such as copepods, rotifers and *Artemia*. Live feeds are expensive, time-consuming, labor intensive, unreliable and nutritionally imperfect. An alternative to live feeds is microparticulate feeds, however, high performance microparticulate diets for larval finfish are typically closed formula commercial diets that make scientific inference difficult or impossible. We describe a series of open formula microparticulate reference diets that have been formulated and produced to facilitate comparisons across species and systems for use by the scientific community. Open formula diets can produce consistent results among trials and species, they can be simply formulated with well-defined ingredients and provide a basic platform for improvement. The reference diets were processed by three methods: flaking (F), microextrusion followed by marumerization (MEM) and particle-assisted rotational agglomeration (PARA). An additional two diets were made by using the flake diet and further processing it using the MEM (F-MEM) or PARA (F-PARA) methods. The F, F-PARA and F-MEM diets had the same formulation and only differed by processing method. PARA and MEM had unique formulations. Each diet was screened to the 400–700 μm range. All microparticulate diets were compared to enriched *Artemia* and rotifers (*Brachionus plicatilis*) for chemical analysis. Scanning electron microscopy (SEM) was used to visualize the microparticles between 22x and 4000x magnification. It was clear that micropellet structure, sinking rate and leaching was influenced by both formulation and manufacture method. Basic structure remained largely intact for all but the F-PARA particles even after being immersed in fresh water for 15 minutes. Compositional data, leaching half-life and sinking rates are given in the table below (values in a column with differences at $P<0.05$ are denoted by superscripts). Data on feeding trials with larval fish indicate that two of the open formula diets performed as well as, or almost as well as, a popular commercial diet (Otohime, Marubeni Nisshin Feed, Tokyo, Japan) with larval yellowtail (*Seriola lalandi*), white sea bass (*Atractoscion nobilis*) Pompano (*Trachinotus carolinus*) and red drum (*Sciaenops ocellatus*). This performance illustrates that these diets provides a good base on which to make improvements.

Annotated Bibliography

Barrows F. T., and Lellis W. A., 2000: Microbound feeds. In R. R. Stickney (ed) Encyclopedia of Aquaculture. John Wiley & Sons, New York. 525–528.

In addition to a review of methods to make older micro bound feeds for aquaculture, methods to

2015年1月30日受理（Received on January 30, 2015）
*1 NOAA, Northwest Fisheries Science Center, 2725 Montlake Blvd. E. Seattle, WA 98121
E-mail: Mike.rust@noaa.gov
*2 USDA/ARS-Fish Technology Center, 4050 Bridger Canyon Rd, Bozeman, Montana 59715
*3 Hubbs-SeaWorld Research Institute, 2595 Ingraham Street, San Diego, CA 92109

produce microparticulate feeds using micro extrusion and particle assisted rotational agglomeration are explained.

Cho C. Y., Cowey C. B., and Watanabe T., 1985. Finfish Nutrition in Asia: Methodological approaches to research and development. IDRC, Ottawa, Ont. Canada. 154 p.

Step by step instructions to produce zein and carrageenan bound diets developed in Japan are explained in English.

Holt G. J. (editor) 2010: Larval Fish Nutrition. John Wiley & Sons, New York. 435 p.

Nutrition is particularly important in the healthy development of fish during their early-life stages. Understanding the unique nutritional needs of larval fish can improve the efficiency and quality of fish reared in a culture setting. *Larval Fish Nutrition* comprehensively explores the nutritional requirements, developmental physiology, and feeding and weaning strategies that will allow aquaculture researchers and professionals to develop and implement improved culture practices. *Larval Fish Nutrition* is divided into three sections. The first section looks at the role of specific nutrient requirements in the healthy digestive development of fish. The second section looks at the impacts if nutritional physiology on fish through several early-life stages. The final section looks at feeding behaviors and the benefits and drawbacks to both live feed and microparticulate diets in developing fish.

Langdon C. L., 2003: Microparticle types for delivery of nutrients to marine fish larvae. *Aquaculture*, **227**, 259-275.

Good review and discussion of several types of microparticulate diets including complex microparticles.

Ronnestad I., Yufera M., Ueberscchar B., Ribeiro L., Saele O., and Boglione C., 2013: Feeding behaviour and digestive physiology in larval fish: Current knowledge and gaps and bottlenecks in research. *Rev. Aquaculture (suppl. 1)*, S59-S98.

Food uptake follows rules defined by feeding behaviour that determines the kind and quantity of food ingested by fish larvae as well as how live prey and food particles are detected, captured and ingested. Feeding success depends on the progressive development of anatomical characteristics and physiological functions and on the availability of suitable food items throughout larval development. The fish larval stages present eco-morpho-physiological features very different from adults and differ from one species to another. The organoleptic properties, dimensions, detectability, movements characteristics and buoyancy of food items are all crucial features that should be considered, but is often ignored, in feeding regimes. Ontogenetic changes in digestive function lead to limitations in the ability to process certain feedstuffs. There is still a lack of knowledge about the digestion and absorption of various nutrients and about the ontogeny of basic physiological mechanisms in fish larvae, including how they are affected by genetic, dietary and environmental factors. The neural and hormonal regulation of the digestive process and of appetite is critical for optimizing digestion. These processes are still poorly described in fish larvae and attempts to develop optimal feeding regimes are often still on a 'trial and error' basis. A holistic understanding of feeding ecology and digestive functions is important for designing diets for fish larvae and the adaptation of rearing conditions to meet requirements for the best presentation of prey and microdiets, and their optimal ingestion, digestion and absorption. More research that targets gaps in our knowledge should advance larval rearing.

Feed	Moisture (%as fed)	Proximate Composition (% dry weight)			Fatty Acid Concentrations (mg fatty acid/100mg total fatty acids)			Protein Leaching $t_{1/2}$/% lost (sec)/(%)	Sinking Rate (cm/sec)
		Protein	Lipid	Ash	ARA (n-6)	EPA (n-3)	DHA (n-3)		
MEM	5.7	53.8	30.5	8.4	0.4	7.5	5.7	98/23.0	0.84±0.010[a]
PARA	5.3	54.2	29.7	8.9	0.4	7.4	5.6	94/30.2	0.34±0.001[c]
F	9.0	75.9	10.9	7.3	1.0	6.0	15.2	20/44.3	0.33±0.001[c]
F-MEM	13.3	75.6	13.4	7.5	1.0	5.3	13.0	50/41.8	0.78±0.060[a]
F-PARA	11.0	75.6	12.6	7.3	1.0	6.0	15.2	33/47.2	0.61±0.001[b]
Artemia	91.3	NA	15.3	13.7	5.6	12.1	7.1	NA/0.1	NA
Rotifers	90.1	49.6	15.5	17.7	1.5	5.7	23.3	NA	NA

水研センター研報，第40号，79－82，平成27年
Bull. Fish. Res. Agen. No. 40, 79－82, 2015

An Analysis of the Causality between the Market Price of Imported Fishmeal and the Market Price of Marine Farmed Fish

Yoshifumi TAKAHASHI*1

Abstract: The purposes of this paper are to introduce the historical background and price trends of the imported fishmeal market, and to reveal the pattern of causality between prices in the imported fishmeal market and the market prices of yellowtail and sea bream inlanding areas. Granger causality test was used in this analysis.

The results were as follows: 1) there is causality between the price of imported fish meal and the market price of sea bream in landing areas, 2) but there is no causality among the market prices of all combinations without imported fishmeal and sea bream.

Key words: Granger causality test, market price of imported fishmeal, market prices of pisciculture

In Japan, many piscicultural farmers are facing difficulties due to the impact of falling fish prices and the rising cost of feed. These are serious problems because they can result in a great reduction in profits for many farmers. According to statistics produced by the Japanese Corporation of Feed for Pisciculture (Nihon Yougyo Shiryou Kyoukai), the production of feed reached 360,000 tons in 2011, the majority of which was for yellowtail and sea bream. These feedstock were made from raw ingredients such as fishmeal, starch, and fish oil. Fishmeal was especially important, making up 50% or more of the feed content. Therefore, piscicultural farming yellowtail and sea bream always worry about soaring imported fishmeal prices. This research investigates the relationship of price changes in imported fishmeal on the market price of piscicultural products.

The purposes of this paper are to introduce the historical background and price trends of the imported fishmeal market, and to reveal the pattern of causality between prices in the imported fishmeal market and the market prices of yellowtail and sea bream in landing areas. Granger causality test was used in this analysis.

The domestic production of fishmeal and the market price of major farmed fishes

The domestic production of fishmeal came mainly from inshore fisheries in northern Japan between 1959 and 1991 (Fig. 1), and the main ingredient, Pollack, was used on the grounds that it was fresh and suitable for processing. However, the catch of Pollack has decreased year by year from then as a result of increasing demand for fish pastes and the impact of fishery treaties such as the 200 mile exclusive fishing zone. As a result, the amount of imported fishmeal has increased gradually to make up for insufficient supplies of local ingredients in Japan. More than half current fishmeal stocks are imported from three countries in South America

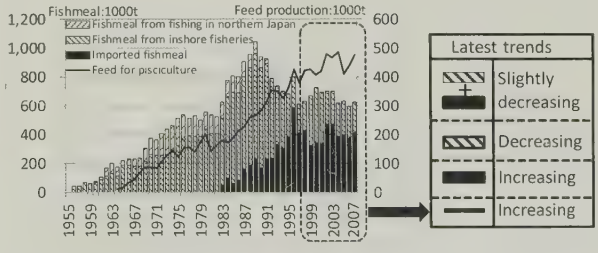
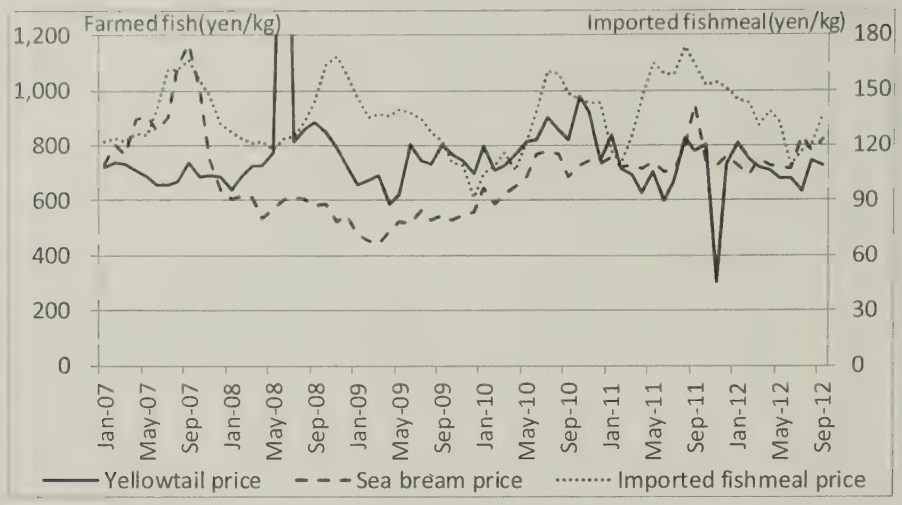


Fig. 1. Domestic production of fishmeal and feed for pisciculture in Japan

2015年1月30日受理 (Received on January 30, 2015)
*1 Tohoku National Fisheries Research Institute, FRA
E-mail: gibun@affrc.go.jp



Note: Those prices were deflated using CPI and IPI (2010=100)

Fig. 2. Market prices for yellowtail, sea bream and imported fishmeal

(Peru, Ecuador and Chile).

Fig. 2 shows the trend of market prices in imported fishmeal, farmed yellowtail and farmed sea bream during the six years from 2007. The market price changes of yellowtail and sea bream have been subject to violent fluctuations within certain limits. As for only yellowtail, there is an exception that the price of yellowtail soared upwards in June 2008 because of little production. Imported fishmeal has also ranged in price from 90 to 175 yen/kg.

Generally, the market price of a commodity tends to rise when the price of ingredients rises; this phenomenon is called "cost push inflation". As seen in Fig. 2, there have been price changes over time, but we cannot conclude that there has been any relationship between changes in the price of one commodity and that of another. Therefore we decided to analyze the relationships among prices using statistical approaches.

Data and Method

Data: Time series data about piscicultural products such as yellowtail and sea bream are available from the website of JAFIC (Japan Fisheries Information Service Center). These original data are nominal, so for this paper these were deflated with "yellowtail" and "sea bream" in the CPI (Consumer Price Index). Also time series data of imported fishmeal are available from the website of the MOF (Ministry of Finance), and have also been deflated with "fishmeal" in the IPI (Import Price Index).

Table 1. Result of the DF-GLS test

	First difference data					
	Yellowtail price			Sea bream price			Imported fishmeal price		
	lag	statistic	significant	lag	statistic	significant	lag	statistic	significant
DF-GLS Test	0	−6.326	**	0	−0.702	*	0	−1.476	*
	1	4.494	*	1	−0.474		1	0.773	
	2	3.803	*	2	−0.612	*	2	0.799	
	3	3.258		3	−0.611	*	3	0.499	
	4	2.817		4	−0.494		4	0.570	
	5	2.401		5	−0.496		5	0.646	
	6	2.004		6	−0.396		6	0.438	
	7	1.620		7	−0.325		7	0.069	
	8	1.249		8	−0.448	*	8	0.229	
	9	0.957		9	−0.356		9	0.305	
	10	0.636		10	−0.216		10	−0.015	
	11	0.323		11	−0.135		11	0.114	
	12	0.119		12	−0.243		12	0.094	

Note: "**" indicates statistical significance at the 1%level, "*" at 5% level

The data first had to be tested for "stationarity" or "not stationarity" before processing in the Granger causality test, because time series data in economics has an almost unit root. Regression among data sets having unit roots has no meaning because of the potential for high correlations among those data. The DF-GLS (Dicky-Fuller is Based on GLS Detrending) test is reliable statistical method to check for unit root, so this was applied. The first test with raw data was not suitable for null hypothesis testing. As a countermeasure for the unit root problem, first difference data was used and then retested in the same way. The result of this retest made it possible for null hypothesis testing to function correctly (Table 1). From this, first difference data whose lag is 0 was adopted.

Analysis method: Analysis method used in this paper

was the Granger causality test in a VAR (Vector Autoregression) model. An AR (Autoregression) single variable model was used, and current value was determined by past own value and white noise (for more information on the VAR model and Granger Causality Test, see chapter 12 and chapter 13 in Maddla (1992)). The VAR model is an expanded AR model, from single to multivariable cases, although the current values of the multivariables are also determined by past own values and white noise. In this section, the two variables VAR(1) model that was actually used is described. The values given in parentheses are lag numbers. Here, x_t and y_t are the price data of commodity at time t, ε denotes error terms, and a_{ij} is an individual parameter.

$$\left.\begin{aligned} x_t &= a_{11}x_{t-1} + a_{12}y_{t-1} + \varepsilon x_t \\ y_t &= a_{21}x_{t-1} + a_{22}y_{t-1} + \varepsilon y_t \end{aligned}\right\} \cdots\cdots(1)$$

The Granger causality test is examined by equation (1). The basic idea of the Granger causality test is that the past value of variable y can affect variable x (or not). It can determine that variable y can affect variable x if the null hypothesis of "$a_{12}=0$" is rejected by F test. The following equation (3) is the form of equation (2) where "$a_{12}=0$".

$$x_t = a_{11}x_{t-1} + a_{12}y_{t-1} + \varepsilon x_t \cdots\cdots(2)$$
$$x_t = a_{11}x_{t-1} + \varepsilon y_t \cdots\cdots(3)$$

The residual sum of squares of equation (2) replaces RSS, the residual sum of squares of equation (3) replaces RSS_0, and we can define the F value using the following equation:

$$F = \frac{(RSS_0 - RSS)/q}{RSS/(n-r-1)},$$

Where n is the number of samples, q is the number of constraint conditions such as "$a_{12}=0$", and r denotes the number of variables without a constant term in equation (2).

Results and Discussion

The VAR model has a problem with respect to lag length selection, so it is necessary to select optimal lag length to obtain an accurate result before applying the Granger causality test. In this paper, the estimation of lag length selection was carried out with four model types which were "none", "const", "trend" and "both". "None" has no drift and trend, "const" has only drift, "trend" has only trend, and "both" has both of these. The estimation of these four types were judged by using S.C. (Schwarz Information Criteria). Consequently, "none" and "lag length = 1" are selected in all combinations of two variable VAR models.

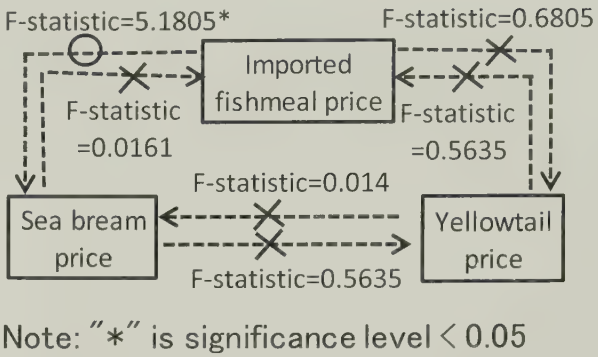


Fig. 3. Result of market linkages in Japan

Fig. 3 shows the results of the Granger causality test. There is no causality of price between yellowtail and sea bream, and this is natural because yellowtail cannot be substituted for sea bream. Also, changes in the price of farmed fish have no influence on the price of imported fishmeal. Why the price of imported fishmeal can affect the market price of sea bream in Japan. We do not have a scientific explanation for the fact that this relationship only affects the price of sea bream. Nevertheless, according to Demura (2010), feed stocks for farmed sea bream are dependent on fishmeal even if the price of imports rises. Conversely, feed stocks for yellowtail can be changed to other ingredients such as raw fish if the price of imported fishmeal rises too high. This situation is probably the explanation.

References

Demura M., 2010: A trend of fishmeal price and impact on the aquacultural industry, *Finance of Agriculture and Forestry*, **45**, 601-605 (in Japanese).

Maddla G. S., 1992: Introduction to Econometrics, 2nd edition, Prentice-Hall, USA.

Japanese Corporation of Feed for Pisciculture, 2007: Pisciculture and formula feed in Japan: A

historical record of 40th anniversary of Japanese Corporation of Feed for Pisciculture, Japanese Corporation of Feed for Pisciculture, Tokyo, Japan (in Japanese).

Yano I., 2010: Heading towards construction of recycling-oriented society associated with food production and consumption: food wastes problem and recycle problem. in Yamao, M. (Ed.), Our "food safety and security" and environmental problem, Hirodai Seibutsuken Shuppankai, Hiroshima, Japan: 83-92 (in Japanese).

水研センター研報，第40号，83－88，平成27年
Bull. Fish. Res. Agen. No. 40, 83－88, 2015

Good Aquaculture Practices to Reduce the Use of Chemotherapeutic Agents, Minimize Bacterial Resistance, and Control Product Quality

Stanley SERFLING*

Abstract: A significant challenge to the expansion of aquaculture production is controlling the outbreak of disease. Many farmers who experience the potential loss of stock from disease may choose to use chemotherapeutic agents to minimize their loss. It is generally understood that a disease in aquaculture is a combination of the health of the animal, the condition of the environment and the presence of a pathogen. From this concept there are a number of precautionary measures that farmers may practice to minimize disease outbreaks. The principles of Hazard Analysis Critical Control Point (HACCP) may be useful risk management tools to reduce pathogens, animal stress and the need for chemotherapeutic agents to control disease outbreaks on the farm.

Key Words: good aquaculture practices, chemotherapeutic agents, bacterial resistance, food safety

Introduction

As the global demand for seafood increases, the trend towards high density aquaculture systems grows. These systems referred to as "intensive aquaculture" increasingly rely on inputs of oxygen, formulated feed, and the application of agrochemicals, and antibiotics. This method of culture may also create stressful conditions for the animals and increase the risk of bacterial infections and therefore the use of chemotherapeutic agents (Sapkota *et al.*, 2008).

Shrimp is the single most popular type of seafood in the United States and over 90 percent of shrimp products are farm raised and imported primarily from Southeast Asian countries. The primary hazards associated with aquaculture shrimp are contamination from pathogens and residues from unapproved drugs. The origins for these hazards are from production farms and can remain in or on the product throughout the normal cleaning, rinsing and packaging process.

Between 2001 and 2003 the U.S. Food and Drug Administration analyzed 1,234 samples from 103 shrimp aquaculture farms representing six countries

Fig. 1.

for fecal coliforms, *Escherichia coli*, and *Salmonella*. A significant relationship was found between the log number of fecal bacteria and the probability that any given sample would contain *Salmonella*. The study concluded the occurrence of *Salmonella* bacteria in shrimp from aquaculture farms is directly related to the concentration of fecal bacteria in the source and production pond water (Koonse *et al.*, 2005).

The importance of chemical and antimicrobial compounds in the protection of animals has been acknowledged, but the negative impact and use of these agents in animals raised for food is a concern.

2015年1月30日受理（Received on January 30, 2015）
* U.S. Food and Drug Administration, Office of Food Safety, College Park, MD 20740
E-mail: stanley.serfling@fda.hhs.gov

The detection of certain banned chemotherapeutic agents in fish and crustaceans in international trade during 2001-2002 led to greater attention about the potential health risks from farm raised seafood products (Iddya, 2012).

Good Aquaculture Practices

Good aquaculture practices (GAqP) can be defined in many ways. In general it is based on preventive practices that are developed and implemented to meet the needs of the species, culture methods and local environmental conditions. When aquaculture producers take time to identify potential risks to their operation and implement controls to manage the risk, then the potential for disease and the use of chemotherapeutic agents is minimized.

The Hazard Analysis Critical Control Point (HACCP) principles can be used as a preventive risk management system to control the introduction of pathogens at aquaculture facilities. The HACCP approach is based on prevention and requires a hazard analysis that identifies a potential hazard in the system and then a Critical Limit, with a maximum and or minimum point, is set for each component of the system. When monitoring the aquaculture system and a critical limit has been in violation, then a corrective action is taken to bring the system back into compliance (Jahncke *et al.*, 2002).

These HACCP principles provide a step by step approach to identify and control hazards found in the environment and production process for both freshwater and marine aquaculture. The following are the seven basic principles with an example:

Fig. 2.

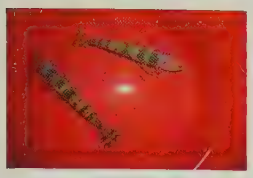
1. Hazard and Risk Analysis: Collect and evaluate information on hazards associated with the product under consideration to decide the likelihood of the occurrence and the severity of the occurrence. To help identify a potential hazard a farmer should construct a flow diagram with all of the inputs to the farm and then evaluate a control for each potential hazard.
   - See Fig. 3 for a shrimp farm flow diagram.

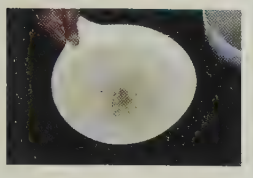
2. Identify Critical Control Point (CCP): This step is essential to prevent or eliminate a hazard or reduce it to an acceptable level.
   - For example a shrimp farmer may choose critical water quality parameters such as minimum and maximum oxygen and temperature levels.

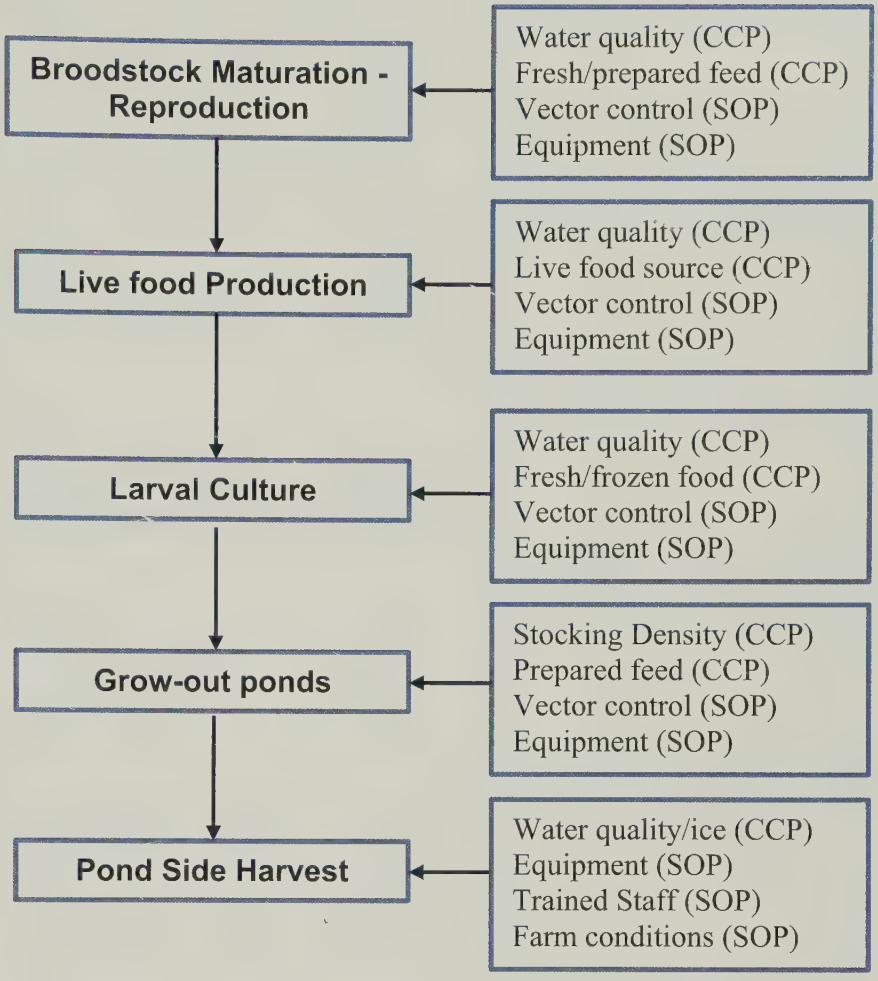
3. Establish Critical Limits: A maximum and/or minimum value to which a physical, chemical or biological hazard must be controlled to prevent, eliminate or reduce to an acceptable level the occurrence of a hazard.
   - For example a critical limit could be stocking density at a hatchery tank and the ratio of live feed to animal.

4. Establish Monitoring Procedures: Monitoring is a process that the aquaculture operation relies on to maintain control of a Critical Control Point. Accurate monitoring is important to help determine when a CCP has deviated from the desired range. Failure to properly use and monitor chemotherapeutic agents may result in detectable residues in the tissue of aquacultured products.
   - For example, monitoring a specific water quality parameter every day or more frequently depending on the production system.

5. Establish Corrective Actions: The farmer should identify procedures to follow when a deviation from the acceptable range occurs.







CCP – Critical Control Point
SOP – Standard Operating Procedure

Fig. 3. Process flow diagram of an integrated shrimp farm with hatchery.

The farmer will implement a corrective action plan after the deviation has been identified and prepare a long term solution as soon as possible.

- For example if a farmer has identified the feed supply for the hatchery does not conform to required standard, the product is rejected and the farmer contacts an alternate feed supplier for new feed.

6. Establish Verification Procedures: This procedure helps to determine the validity of the operation and verify that the farm is operating according to plan.
   - For example the farmer may review daily water quality parameter sand feeding records to determine if the production system is meeting expected output.

7. Establish Record Keeping Procedures: The final step is to document all previous steps, from the Evaluation of Hazards and flow diagram, the identification of Critical Control Points and Critical Limits, and any Corrective Actions taken, and all Verification Procedures.
   - Records should be made for all identified Critical Control Points and be accessible to staff in paper or electronic format.

Fig. 3 is an example of an operational flow diagram for an integrated shrimp farm with brood stock, hatchery, nursery, and growout production.

Preventive Measures

A standard operating procedure (SOP) manual is an important document the farmer should assemble. This should include facility design and flow diagram, general husbandry procedures for each step in the production process, restricted access areas, waste management, pest control and staff rules. Good Aquaculture Practices may also include welfare of the animals, environmental integrity and social responsibility.

There are a number of biological, chemical and physical precautionary measures that can be undertaken. The following are examples of how to apply these practices on the farm.

Biological: Includes measures to prevent or treat infections such as the proper use of chemotherapeutic agents or the use of vaccinesas well as management practices to prevent the spread of pathogens by isolating and testing incoming seed stock. In addition, providing clean water and sanitary facilities for staff and proper farm security help to control the spread of pathogens from humans and animals.

Chemical: Includes measures that are used to prevent the introduction of pathogens or vectors by treating materials before they enter the facility. For example, chlorination or ozone can be used to treat incoming water, and iodine and chlorine can be used to treat other potential vectors such as tools, footwear and clothing.

Physical: Includes barriers that may be useful to prevent vectors from contaminating a farm site. Physical barriers can be stress reducing practices like proper stocking densities, optimal levels for dissolved oxygen and temperatures, and proper feeding practices.

Table 1. The following hazard analysis for an intensive marine shrimp farm.

Identify Potential Hazard	Is the Hazard Significant	Justification	Preventive Measures	CCP
Seed stock	Yes	Shrimp larvae may contain detectable pathogen	SPF certification for each receipt. Quarantine procedures and test periodically (CCP)	Yes
Water Source	Yes	Potential pathogens	Provide pre-treatment to ensure water supply is safe (CCP)	Yes
Feed Source	Yes	May result in and poor production	Certificate from feed supplier to ensure standard (CCP)	Yes
Stocking Density	Yes	Important to optimize production	Controlled by Standard Operating Procedure (SOP)	Yes and No
Effluent or Recirculated Water	Yes	Effluent may contain pathogens	Controlled by Standard Operating Procedure (SOP)	Yes and No
Animal and Human Vectors	Yes	May transfer contaminants and pathogens	Controlled by Standard Operating Procedure (SOP)	No
Farm Equipment and Sanitation	Yes	Equipment may become contaminated with pathogens	Controlled by Standard Operating Procedure (SOP)	No

SPF – Specific Pathogen Free
CCP – Critical Control Point
SOP – Standard Operating Procedure

Conclusion

Good Aquaculture Practices may be applied to many different kinds of aquaculture systems in a variety of management strategies. The key elements can be summarized as reliable sources of stock, good diagnostic and detection methods for disease, disinfection and eradication of pathogens and best management practices. HACCP principles provide a logical step by step approach to help prevent pathogen contamination and the outbreak of disease on the farm site. This management approach allows the farmer to choose the most important process control steps and then choose appropriate critical control points and control measures to minimize risk to the animals and the farm operation and insure that chemotherapeutic agents if needed will be used wisely.

References

Jahncke M. L., Browdy C. L., Schwarz M. H., Silva J. L., Smith D. C., and Stokes A. D., 2002: Risk management, disease prevention, and HACCP principles: Application of hazard analysis critical control point (HACCP) principles as a risk management tool to control viral pathogens at shrimp aquaculture facilities. NOAA Office of Sea Grant, Virginia Graduate Marine Science Consortium and the Virginia Sea Grant College Program, Publication VSG-02-10, Pages 1-33.

Karunasagar I., 2012: Public health and trade impact of antimicrobial use in aquaculture, in "Improving biosecurity through prudent and responsible use of veterinary medicines in aquatic food production" (ed by Bondad-Reantaso M. G., Arthur J. R. and Subasinghe R.P.) FAO Fisheries and Aquaculture Technical Paper No. **547**. Rome, FAO, pp. 1-9.

Koonse B., Burkhardt W., Chirtel S., and Hoskins G., 2005: Food safety and aquaculture, controlling risk, coliform bacteria: Salmonella and Sanitary Quality of Aquaculture Shrimp. *J. Food Prot.*, **68**, No 12, 2527-2532.

Sapkota A., Sapkota A., Kucharski M., Burke J., McKenszie S., Walker P., Lawrence R., 2008: Aquaculture practices and potential health risks: Current knowledge and future priorities. *Environ. Int. J.*, **34**, 1215-1226.

Annotated Bibliography

Jahncke M. L., Browdy C. L., Schwarz M. H., Segars A., Silva J. L., Smith D. C., Stokes A. D., 2002: Risk management, disease prevention, and HACCP principles: Application of hazard analysis critical control point (HACCP) principles as a risk management tool to control viral pathogens at shrimp aquaculture facilities. NOAA Office of Sea Grant, Virginia Graduate Marine Science Consortium and the Virginia Sea Grant College Program, Publication VSG-02-10, Pages 1-33.

The authors identify how the principles of HACCP (Hazard Analysis Critical Control Point) can be used as a preventive risk management system to control the introduction of pathogens and the outbreak of disease in shrimp aquaculture facilities. The HACCP approach requires a hazard analysis that identifies a potential hazard in the system and then a Critical Limit with a maximum and or minimum point is set for each component of the system. When monitoring the aquaculture system and a critical limit has been in violation, then a corrective action is taken to bring the system back into compliance.

To develop an effective biosecurity program fish and shrimp farmers should follow these principles based on seafood HACCP: 1) Perform Systematic Hazard Analysis, 2) Determine Critical Control Points, 3) Establish Critical Limits, 4) Determine Appropriate Corrective Actions, 5) Establish Monitoring Procedures, 6) Establish Verification Procedures, 7) Establish Record Keeping Systems.

Karunasagar I., 2012: Public health and trade impact of antimicrobial use in aquaculture, in "Improving biosecurity through prudent and responsible use of veterinary medicines in aquatic food production" (ed by Bondad-Reantaso, M.G., Arthur J. R. and Subasinghe R.P.) FAO Fisheries and Aquaculture Technical Paper No. **547**. Rome, FAO,pp. 1-9.

The Food and Agriculture Organization of the United Nations (FAO), the World Health

Organization (WHO) and the World Organization for Animal Health (OIE) organized consultations and technical meetings to review the global seafood trade and the use of antimicrobial agents in aquaculture and develop recommendations. The author outlines how detection of certain banned antibiotics in fish and crustaceans in international trade during 2001–2002 lead to greater attention on the public health risks owing to the use of antimicrobial agents in aquaculture. Most fish importing countries adopt a zero tolerance approach regarding residues of antimicrobials that are banned for use in food animals. In such cases, residue levels that attract regulatory action are based on analytical capability rather than toxicology of the residues.

Koonse B., Burkhardt W., Chirtel S., and Hoskins G., 2005: Food safety and aquaculture, controlling risk, coliform bacteria: Salmonella and Sanitary Quality of Aquaculture Shrimp. *J. Food Prot.*, **68**, No 12, 2527–2532.

The authors examined the prevalence of *Salmonella* and coliform bacteria on shrimp aquaculture farms in several Asian countries to develop guidelines or preventative measures for reducing *Salmonella* and fecal contamination on products harvested from these farms. A significant relationship was found between the log number of fecal bacteria and the probability that any given sample would contain *Salmonella*. The likelihood of finding *Salmonella* in grow-out pond water increased 2.7 times with each log unit increase in fecal coliform concentration and 3.0 times with each log unit increase in *E. coli* concentration. *Salmonella* is not part of the natural flora of the shrimp culture environment nor is it inherently present in shrimp grow-out ponds. The occurrence of *Salmonella* bacteria in shrimp from aquaculture operations is related to the concentration of fecal bacteria in the source and grow-out pond water.

Sapkota A., Sapkota A., Kucharski M., Burke J., McKenszie S., Walker P., Lawrence R., 2008: Aquaculture practices and potential health risks: Current knowledge and future priorities. *Environ. Int. J.*, **34**, 1215–1226.

The authors conducted an extensive literature search with 145 peer reviewed papers to identify current global aquaculture practices and the potential health risks from aquacultured seafood. The authors focused on aquaculture production in Asia as having 11 of the top 15 producing countries in the world. The paper identifies chemical and biological contaminants used by the aquaculture industry and the potential impacts on food safety and human health. Tables identify the top 15 countries that use antibiotics and the prevalence of antibiotic resistance in the environment as a potential health concern. As global aquaculture production continues to increase worldwide with an emphasis on intensive systems, exposure to various hazardous chemical and biological compounds is a concern. The authors conclude the increased use of chemotherapeutic agents is contributing to elevated levels of antibiotic residues and antibiotic resistant bacteria.

水研センター研報，第40号，89－96，平成27年
Bull. Fish. Res. Agen. No. 40, 89－96, 2015

Modeling Intraspecific Genetic Effects for Management of Aquaculture Programs

Jason D. VOLK[*1], Michael B. RUST[*2], Gregory R. BLAIR[*1], Lars E. MOBRAND[*1], Conrad V. W. MAHNKEN[*3], and Walton W. DICKHOFF[*2]

Abstract: Rapid worldwide development of marine finfish cage farming has raised awareness over the possible genetic and ecological effects of escaped fish on wild populations. With increased interest in implementation of marine aquaculture in the United States, NOAA Fisheries and other regulators charged with stewardship of marine ecosystems need tools to understand and mitigate risks presented by aquaculture escapees. To develop an understanding of genetic and ecological effects of escapes and design management strategies to address potential risks to marine resources, NOAA Fisheries has developed a numerical decision-support tool: the Off-shore Mariculture Escapes Genetics/Ecological Assessment (OMEGA) model. The OMEGA model is an extension of concepts from another model, the All-H Analyzer (AHA) that is used successfully in the U.S. Pacific Northwest to evaluate genetic and ecological interactions between hatchery and wild salmon and trout.

OMEGA model input parameters include size and growth characteristics of cultured fish, frequency and magnitude of escape events, survival of escapees in the wild, probability of escapees encountering a conspecific natural population and interbreeding, and population dynamics of the natural population. Model results describe the influence of aquaculture escapees on spawning biomass, juvenile production, and genetic fitness of the composite population. Effects of interactions on fitness and abundance are based on the frequency and relative abundance of cultured fish that escape and survive to encounter a natural population, the difference in survival characteristics between the artificial and the natural environments, and the genetic legacy of the cultured and natural populations.

NOAA Fisheries is using the OMEGA model to identify and evaluate risks of marine aquaculture operations, design sustainable aquaculture programs, explore the effects of regulation, and identify research priorities for areas of uncertainty.

This talk will describe the model and present results for a hypothetical sablefish (*Anoplopoma fimbria*) culture program along the U.S. West Coast. We are interested in speaking with any and all individuals interested in collaborating on the further development of the model, applying the model to other species of interest such as rockfish (*Sebastes* spp.), yellowtail (*Seriola quinqueradiata*), salmon (*Oncorhynchus spp.*), or any other aquaculture candidate species, and to identify opportunities to validate model results.

Key words: escapes, survival, introgression, simulation, fitness, selection, OMEGA.

2015年1月30日受理（Received on January 30, 2015）
*1 ICF International, 710 Second Avenue Suite 550, Seattle, WA 98104, U.S.A.
*2 NOAA Fisheries, Northwest Fisheries Science Center, 2725 Montlake Blvd. East, Seattle WA, 98112, U.S.A.
*3 Aquatic Resource Consultants, 9933 NE Lafayette, Bainbridge Island WA 98110, U.S.A.
Corresponding author: Jason D. Volk E-mail jason.volk@icfi.com

Modeling intraspecific genetic effects for Management of Aquaculture Programs

The development of world cage farming of marine fishes has raised concerns over the possible genetic and ecological impact of escaped fish on wild populations. Marine fish can escape from farms for a variety of reasons including: when improper mesh size is used or when holes in webbing develop from normal wear and tear, during transfer from cage to cage or while grading or harvesting fish, from high wind and high sea conditions during severe storms, or when net cages are breached by large predators. Potential effects include the introduction of maladaptive genes and reduced fitness, competition for food and space, and predation on native stocks.

Several factors merit consideration when evaluating the likely ecological and genetic impacts cultured fish may have should they escape. Among them are: the wild population genetic structure and phenotypic variability of a species or among stocks within a species, the size of the local (or affected) population relative to the estimated number and frequency of escapement, the type of breeding program to be used, including selection of the founding stock, and the likelihood that unintentional genetic drift (domestication) related to hatchery practices will occur.

In reality, there is little evidence-based information available that can reliably assign risk to escapes of finfish from aquaculture facilities, therefore, escape standards are, out of necessity, more preventative than prescriptive. Because of uncertainty, locally adapted indigenous species will probably be encouraged over the use of nonnative and genetically modified species unless a compelling argument can be made that the genetic and ecological risks are demonstrably low.

The Offshore Mariculture Escapes Genetic Assessment (OMEGA) Model was developed by ICF International (ICF) and NOAA as a tool for use by scientists and resource managers to help with understanding the potential negative impact of farmed escapees on their wild counterparts.

The purpose of OMEGA is to identify and weigh environmental risks of escapes of marine aquaculture fish to their wild conspecifics. OMEGA is intended to: 1) provide insights about factors affecting risks associated with escapes from aquaculture operations, 2) help identify research priorities, 3) explore options for the design of sustainable aquaculture programs, and 4) inform policy and management decisions related to the genetic and ecological risks of aquaculture.

OMEGA characterizes the aquaculture program by brood source, size and growth of cultured fish, and frequency and magnitude of escape events. Genetic and ecological interactions are calculated from assumptions regarding survival of escapees in nature, their likelihood of encountering conspecifics, breeding success, and the consequence of interbreeding on the fitness and abundance of wild conspecifics. The 100-year model simulation describes the influence of aquaculture escapees on survival and genetic fitness of the natural population. By evaluating different aquaculture operation scenarios and wild stock population dynamics, OMEGA allows the user to compare differences in total abundance trends of escapees and wild fish, as well as the effects of aquaculture program on survival and long-term sustainability of wild fish (ICF 2014).

The modeling concepts of OMEGA have been successfully applied over the past decade using a similar model, the All-H Analyzer (AHA). AHA has been successfully used by the Hatchery Scientific Review Group (HSRG) to evaluate 178 salmon and trout hatchery programs in the Columbia River Basin (Paquet *et al.*, 2011). Using model simulation results, the HSRG formulated a working hypothesis for baseline conditions from which they evaluated strategies to better achieve stated goals for hatcheries and wild populations. The HSRG concluded that through following recommended management and harvest practices, such as broodstock selection and selective harvest, hatcheries can serve dual goals of contributing to harvest while remaining compatible with or contributing to conservation goals. The guiding principles of the HSRG are core to the purpose of the OMEGA model.

OMEGA Model Structure

As shown in Fig. 1, the OMEGA input data structure is organized around three components:

- the biology and operations of the cultured population, including number of fish by size classification, broodstock source (domesticated or wild), maturation and growth factors;
- the wild conspecific population, including assumptions of abundance, distribution, survival, age and size at maturity, age composition, and age specific harvest rates; and
- factors affecting the potential for interaction between farmed fish escapees and their wild conspecifics, assumptions of frequency and magnitude of fish escaping from the pens, survival of escapees in nature, location of the mariculture pens relative to the wild population, potential movement patterns of escapees and breeding success of escapees in nature.

OMEGA accounts for the different factors by which fish can escape from cages, such as:

- *Chronic leaks* from cages, which can be considered a baseline level of escape from year to year, assuming there are a certain amount of fish lost due to routine factors such as small tears in nets, transfer between cages, or veterinary maintenance;
- *cage failures* due to a mechanical failure or defect in a cage containing one size class of fish (this can also occur because cage farms attract predators);
- *catastrophic events* due to failure of multiple cages in the farm structure, resulting in a massive loss of fish, such as from a major storm event; and
- spawning in cages, leading to release of *gametes*.

Once cultured fish escape into nature, their survival is described by a number of factors, such as size at time of escape, level of domestication and their ability to survive in nature, and environmental factors. An attrition of the escapes occurs in the model simulation based on these factors, resulting in a fraction of escapees surviving to encounter wild-origin fish. If escapees successfully reproduce in the wild, there are spawners in nature persisting with potentially maladapted traits. There leads to an effect on genetic fitness of wild fish, which

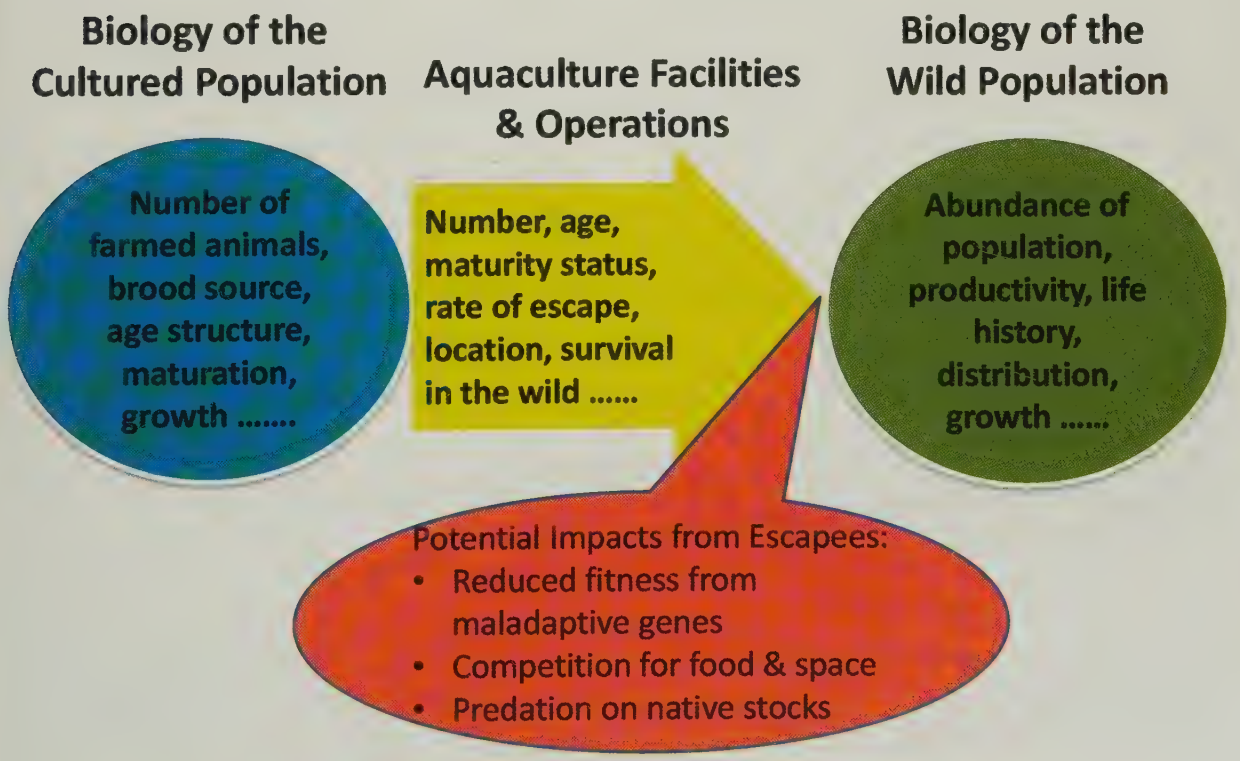

Biology of the Cultured Population
Aquaculture Facilities & Operations
Biology of the Wild Population
Number of farmed animals, brood source, age structure, maturation, growth .....
Number, age, maturity status, rate of escape, location, survival in the wild .....
Abundance of population, productivity, life history, distribution, growth .....
Potential Impacts from Escapees:
• Reduced fitness from maladaptive genes
• Competition for food & space
• Predation on native stocks

Fig. 1. OMEGA Model Components

consequently affects wild recruitment and harvest.

Included in this module are parameter inputs for the phenotypic fitness model in OMEGA. The equations are those described by Ford (2002) and described in additional detail by Campton (2009) for evaluating relative fitness effects of hatchery salmonids interbreeding with wild populations. The phenotypic trait model is described by two environmental optimum values, an aquaculture optimum and a natural optimum, with selection forcing the respective populations towards their environmental optimum (Fig. 2). The model calculates the shift in phenotype trait value of the wild population based on assumed gene flow between escapees and wild fish. The consequence is a loss in fitness and survival of the wild population as a result of this mixed wild population (the progeny of escapees and wild parents) phenotype.

OMEGA Model Simulation Example

This example uses a hypothetical commercial aquaculture operation for sablefish (*Anoplopoma fimbria*) along the coast of Washington, Oregon and California. The purpose of this example is to show how escapes can affect population abundance and long-term viability, and how limiting escapes can greatly reduce impacts to fitness and abundance of the wild population. These scenarios were developed to demonstrate model capabilities and to test model sensitivity to changes in parameter values, and are not intended to describe the operations of an actual sablefish aquaculture industry, or to suggest standards that should be used to regulate operations; they are provided only to describe the functions of the model.

In nature, sablefish reach maturity at 6 years of age and can live up to 80 years. Fish are fully selected to the fishery at 4 to 5 years of age. In culture, sablefish can grow to a harvestable size of 1 kilogram in approximately 52 weeks. In this example, the culture program is assumed to consist of 50 separate offshore pen operations, each containing multiple pens with different size classifications as fish grow to harvestable size. Total culture production is assumed to be 10,000 metric tons per year. For the two scenarios described below, natural production, harvest, culture production, encounter rate, and fitness and interactions including genetic effects are the same. Only escape rates were changed between scenarios (chronic losses, cage failures, and catastrophic events).

In the "low escape scenario", catastrophic events could occur with a probability of 5% in a given year for the first 25 years. After year 25, it is assumed technology improvements would reduce the probability of catastrophic events (1%) resulting in a loss of up to 20% of cultured stock. The scenario assumes that chronic losses are the same across all fish sizes (0.1% leakage). Cage failure rate is assumed to be the same across all size categories (0.1%). Chronic losses and moderate events together average about 200,000 fish escaping per year.

In the "high escape" scenario, catastrophic events are assumed to occur with a probability of 5–10% on a given year and each event results in up to a 60% loss of cultured stock. Chronic losses depend on fish size classification (3% for the smallest size class, 0.5%

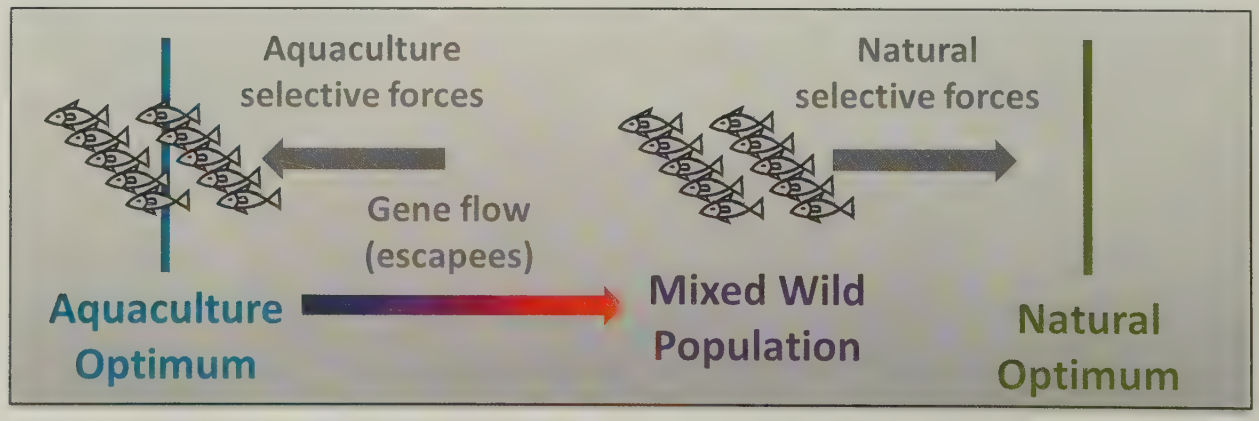


Fig. 2. The Ford Model: Two Environments, Two Selective Forces

for harvest size fish). Cage failure rate is assumed to be 0.5% for smallest size class and 1.0% for harvest size fish. Chronic losses and moderate events together average about 1.0 million fish escaping per year. This is an extremely high percent of total production (10.0 million fish at harvest size) and would represent an extreme example of escapes due to chronic and moderate events. In the 100-year simulation, five random catastrophic events occurred resulting in even larger losses.

The model response in terms of natural population fitness under both scenarios is shown in Figure 3. Escape rates in "low escapes" resulted in a slight shift in the phenotypic trait value of the wild population toward the aquaculture optimum value over the 100-year simulation.

Consistent with the slight shift in trait value, relative fitness also declined slightly, indicating effects of introgression. However, natural fitness reaches an equilibrium value fairly quickly (~year 25). In contrast, under the "high escapes" scenario, natural fitness responded quickly to the effect of escapes on the wild population and it appears fitness does not approach a lower equilibrium value until year 75.

The response of the wild population in terms of mixed population natural recruits under both scenarios is shown in Figure 4. The dark line represents no-escape scenario (i.e. 0% change) as reference. Under the "low escapes" scenario, there is an initial increase in recruitment (progeny of escapee and wild parents) due to increases spawning biomass relative to no escapes, with the value approaching an equilibrium value of about +0.5% at year 100 of

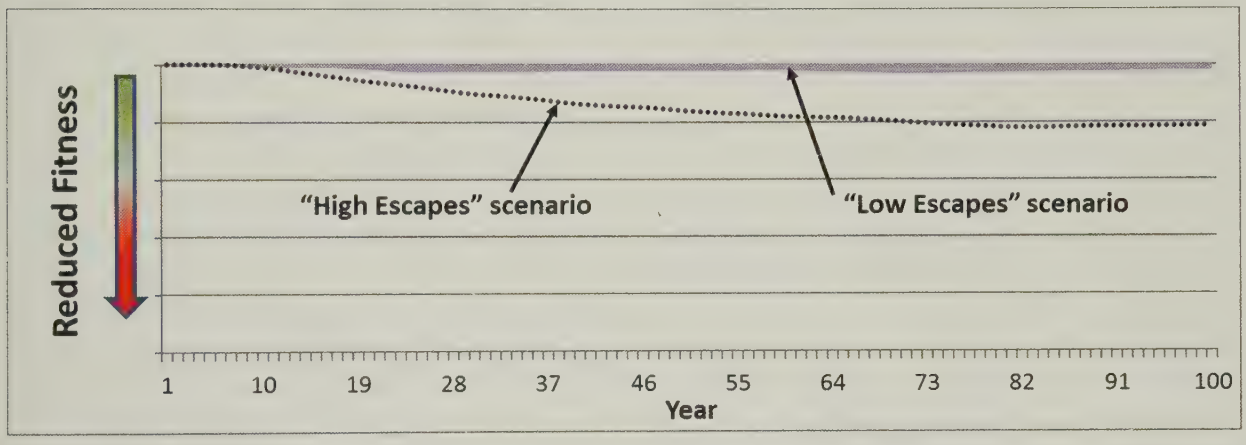


Fig. 3. Natural Population Fitness, 100-year simulation

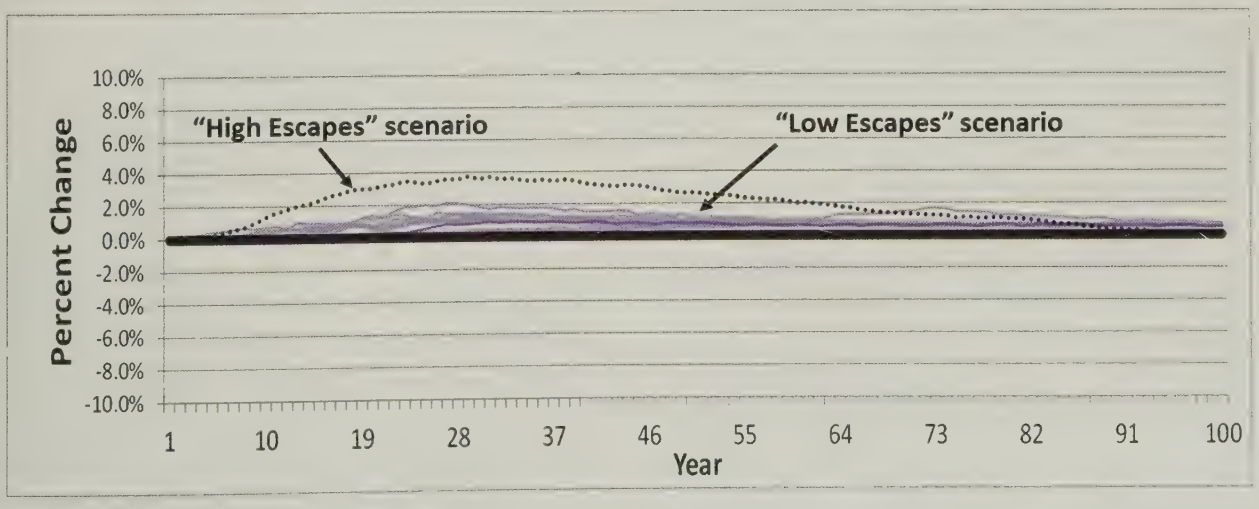


Fig. 4. Mixed Population Natural Recruits, 100-year simulation

the simulation.

The "high escapes" scenario describes the model response if there was greater selective pressure on the population due to a high level of escapes in nature. As in the previous scenario, there is an initial increase in recruitment; more than in the previous scenario but the decline is steep and nearly constant after year 30, approaching negative values in year 100. This trend would likely continue for some years due to a long-term loss in natural fitness.

The results suggest a lag between natural recruitment (Fig. 4) and fitness (Fig. 3). Initially the trend is increasing recruitment while fitness was declining. This lag is likely due to the retention of older, larger and fit adults in the spawning biomass. As more fit fish die out over time the population is shifting to less fit adults and the consequence is a decline in wild production. The results of the "high escape" scenario indicate year-over-year declining fitness and reduced survival of wild fish. The long-lived nature of sablefish is a factor in the delayed effect on wild fish biomass.

OMEGA includes many other parameter inputs that allow for exploration of different scenarios and evaluate model sensitivity due to combinations of parameter value changes (culture and wild population).

Balancing aquaculture goals with conservation goals will require careful evaluation of the risks and benefits. The challenge of decision makers is to find solutions that serve both goals. The purpose of OMEGA is to help identify possible solutions, or if a range of solutions exists, how programs can be managed for the best outcomes.

Next Steps

OMEGA is ready for general use. The model is available from NOAA along with a user guide that includes model background and instructions for using the model. The model also includes the scenarios for sablefish discussed in this presentation. We are interested in your feedback and comments. At this time we seek collaborators to develop case studies for currently farmed or considered aquaculture finfish species. Items for future development include completing a sensitivity analysis of scenarios for sablefish or other species, and developing an economic cost/benefit analysis module.

OMEGA is available at:
http://www.nmfs.noaa.gov/aquaculture/science/26_omega_model_homepage.html

References

Campton D. E., (Hatchery Scientific Review Group), 2009: Columbia River hatchery reform system-wide report. February 2009 Appendix A. 278 p. Available: <http://www.hatcheryreform.us/hrp_downloads/reports/columbia_river/system-wide_full/columbia_river_hatchery_reform_system-wide_report.zip>.

Ford M. J., 2002: Selection in captivity during supportive breeding may reduce fitness in the wild. *Conservation Biology*, **16**, 815–825.

ICF International., 2014: Offshore Mariculture Escapes Genetic/Ecological Assessment (OMEGA) Model Version 1.0 Model Overview and User Guide. August. Prepared for NOAA Fisheries, Seattle, WA.

Paquet P. J., Flagg T., Appleby A., Barr J., Blankenship L., Campton D., Delarm M., Evelyn T., Fast D., Gislason J., Kline P., Maynard D., Mobrand L., Nandor G., Seidel P., and Smith S., 2011: Hatcheries, Conservation, and Sustainable Fisheries—Achieving Multiple Goals: Results of the Hatchery Scientific Review Group's Columbia River Basin Review, Fisheries, **36:11**, 547–561.

Annotated Bibliography

Jensen Ø., Dempster T., Thorstad E. B., Uglem I., and Fredheim A., 2010: Escapes of fishes from Norwegian sea-cage aquaculture: causes, consequences and prevention. *Aquaculture Environment Interactions*, **1**, 71–83.

The authors broadly discuss the magnitude of the problem of escapes from salmon and cod cage-farming aquaculture operations in Norway and provide specific recommendations to prevent escapes. Current knowledge about the extent of threats presented by escapes in terms of economic

and ecological impacts are discussed in the context of experiences in the Norwegian aquaculture industry. While escapes occur due to several internal and external factors, reports from fish farming companies indicated that cage failure was by far the most common cause of large-scale escape in Atlantic salmon farming operations. They report studies that show the mechanisms of escape are not the same across species. Atlantic cod may cause more wear to nets and be more likely to escape through tears in the net. Consequences of escape, such as disease transfer, interbreeding, competition, and predation are generally discussed as areas for further research. The main message of this paper is that prevention is the best tool to reduce the risk of escapes. The authors report evidence that the level of escapes from cages was greatly reduced in Norway following legislation that has specific requirements for design of farms and the handling and use of equipment. They recommend countries develop similar measures to reduce escapes such as mandatory reporting of escapes, a process to use these reports to develop better standards, mandatory technical assessments following reported "large" escape events, technical standards for equipment, and finally, identification of key operational components that have a higher potential to cause an escape event, including training of staff to reduce human errors. This paper was used to identify mechanisms of escape through cage failure and operational scenarios, which are central to development of the escapes component of the OMEGA simulation model.

Meager J. J., Sklæraasen J. E., Fernö A., and Løkkeborg S., 2010: Reproductive interactions between fugitive farmed and wild Atlantic cod (*Gadus morhua*) in the field. *Canadian Journal of Fisheries and Aquatic Sciences*, **67**, 1221-1231.

The authors present a study of spawning interactions between cultured and wild Atlantic cod and tested the potential for hybridization between farm escapes and wild conspecifics. Using a spatial and temporal analysis of wild and farmed cod tracked through biotelemetry, positioning of fish based on sex and origin indicated that farmed fish behave differently from wild fish relative to spawning ground location. However, despite these differences, hybridization is likely, especially between farmed females and wild males. The results illustrate that behavioral differences between cultured and wild fish may not preclude spawning interactions. The authors conclude there is a high potential for farmed cod to hybridize with wild fish. They recommend further research should be a priority to further understand the consequences of interbreeding and to identify methods for escape prevention.

Ford M. J., 2002: Selection in captivity during supportive breeding may reduce fitness in the wild. *Conservation Biology*, **16**, 815-825.

Much of the concern surrounding effects of escaped cultured fish involves interbreeding with wild conspecifics and potential loss of genetic fitness of the wild population. Ford presents a single trait phenotypic model that assumes different optimum trait values for the culture and natural environments. The Ford model describes how mean phenotype values of captive and wild fish shift relative to optimum values for the environments based on gene flow between escapees (or captive breeding) and wild fish. The results suggest that cultured fish can have a strong influence on the fitness and sustainability of wild populations depending on the amount of interbreeding. The level of effect depends on the details of the model such as differences in optimum trait value, selection pressure, and trait heritability. Controlling gene flow between wild and cultured fish can potentially reduce the domestication effect in wild populations. Overall outcomes of fitness in the wild also depend heavily on habitat capacity and population dynamics. This model has been used in several other studies to explore the potential consequences to wild population fitness from captive breeding to increase the size of wild populations, and from the unintended straying of cultured fish to wild populations.

Paquet P. J., Flagg T., Appleby A., Barr J., Blankenship L., Campton D., Delarm M., Evelyn T., Fast D., Gislason J., Kline P., Maynard D., Mobrand L., Nandor G., Seidel P., and Smith S., 2011: Hatcheries, Conservation, and Sustainable Fisheries—Achieving Multiple Goals: Results

of the Hatchery Scientific Review Group's Columbia River Basin Review. *Fisheries*, **36**: 11, 547–561.

The Hatchery Scientific Review Group (HSRG) was established by the U.S. Congress to review salmon and trout hatchery programs in the Pacific Northwest with the goal of recommending hatchery reform guidelines while still retaining the goal of providing fish for harvest and conservation goals for natural populations. This paper presents the approach used and recommendations that included an assessment of 178 hatchery programs and 351 salmonid populations within the Columbia River Basin. This approach included a scientific framework and three principles to guide their assessment: 1) "clear and specific quantifiable goals for harvest and conservation," 2) "be scientifically defensible," and 3) "include monitoring and evaluation of benefits and risks." HSRG used the All-H Analyzer (AHA) model to evaluate dynamics of populations in the Columbia River system through an integrative analysis of several factors related to hatchery operations, and productivity and capacity of wild populations. Using model simulation results, HSRG formulated a working hypothesis for baseline conditions and to evaluate strategies to better achieve stated goals for hatcheries and wild populations. The HSRG concluded that through following recommended management and harvest practices, such as broodstock selection and selective harvest, hatcheries can serve dual goals of contributing to harvest while remaining compatible with or contributing to conservation goals. The guiding principles stated by the HSRG are the core purpose of the OMEGA model.

水研センター研報，第40号，97－107，平成27年
Bull. Fish. Res. Agen. No. 40, 97－107, 2015

Application of Physiological Tests to Determine Specific Monovalent and Divalent Ion Supplementation for Culture of Marine Species

Calvin FISHER*1, Charlotte BODINIER*2, Adam KUHL*3, and Christopher C. GREEN*1

Abstract: The culture of marine and euryhaline fishes in low salinity or ion deficient waters has been an area of interest for many aquaculturists due to the expense of marine salt mixes. The Gulf killifish (*Fundulus grandis*) is a euryhaline teleost found abundantly in coastal marshes along the Gulf of Mexico. The ability to investigate the molecular underpinnings of specific ions in this model species could have a number of implications for other commercially important marine finfish species. Although studies have examined the influence of salinity on adults and juveniles, few have investigated the role of salinity or specific ion concentrations in larvae. These investigations utilize a model teleost to determine the role of specific monovalent and divalent ions at biochemical and molecular levels

Separate four week trials were conducted exposing newly hatched Gulf killifish to concentration gradients of potassium (K^+), calcium (Ca^{2+}), and magnesium (Mg^{2+}). The K^+ supplementation consisted of 0.3, 1.3, and 2.9 mM. Treatment groups for Ca^{2+} consisted of 0.2, 1.1, 1.5, and 2.1 mM Ca^{2+}, while trials using Mg^{2+} consisted of 0.1, 2.7, 5.1, and 10.4 mM Mg^{2+}. All treatments were maintained at a salinity of 9.5–10‰ using crystal salt (99.6% NaCl). Each investigation consisted of four 50-L aquariums stocked at 7 larvae per liter for each concentration. Fish were sampled at 0, 1, 3, 7, 10, 14, and 28 d post hatch (DPH). Upon each sampling the standpipe was adjusted to maintain a constant density for each treatment. Collected samples were analyzed for whole body ion concentrations (K^+, Na^+, Mg^{2+}, Cl^-), Na^+/K^+-ATPase (NKA) activity, dry weight, and expression/localization of ion transport proteins (NKA, $Na^+/K^+/2Cl^-$ cotransporter (NKCC) and cystic fibrosis transmembrane conductance regulator (CFTR)).

Mortality and growth was significantly influenced by K^+ concentration ($P < 0.05$). No differences were observed among treatment groups for NKA enzyme activity, however at 28-d post hatch (dph) there were significant differences in dry weight among K^+ treatment. At seven dph differences in intestinal NKA and CFTR staining were observed, and NKA mRNA expression was also found to be higher in the 0.3 mM [K^+] group than in other treatment groups. Survival was significantly influenced by both Mg^{2+} and Ca^{2+} concentration ($P \leq 0.05$). Highest survival (71.1%) in the Ca^{2+} trial was noted in the 0.2 mM [Ca^{2+}] treatment. In the Mg^{2+} trial, highest survival was noted in the 2.7 mM [Mg^{2+}] treatment (82.9%). NKA enzyme activity was reduced and delayed peaks were observed in whole body ion composition in the Mg^{2+} treatments. In addition, decreased CFTR intensity was observed at the gill and intestine epithelium for the 0.05 mM [Mg^{2+}] treatment at 1 dph, indicating that Mg^{2+} deficiency has a possible effect on larval osmoregulatory capability. The role of intestinal epithelium in ion uptake not only allows the potential for uptake and maintenance of ion balance from dietary sources, but also demonstrates the potential to modulate concentrations of specific ions in prepared waters for euryhaline and marine teleosts.

Key words: Gulf killifish, osmoregulation, potassium, magnesium, euryhaline

2015年1月30日受理（Received on January 30, 2015）
*1 Aquaculture Research Station, Louisiana State University Agricultural Center, Baton Rouge, LA, USA
*2 Rosenstiel School of Marine & Atmospheric Science, Miami, FL, USA
*3 Huntsman Corporation, Houston, TX, USA
Corresponding author:Christopher C. GREENE-mail: cgreen@agcenter.lsu.edu

The major ions in seawater are; chloride (Cl^-), sodium (Na^+), magnesium (Mg^{2+}), Calcium (Ca^{2+}), sulfate ($SO4^-$), and potassium (K^+), with a variety of other ions present in low concentrations. The three ions Ca^{2+}, Mg^{2+}, and K^+ are necessary for maintaining electrolyte and acid-base homeostasis, and the regulation of osmolality and intracellular fluids (Fielder *et al.*, 2001). The high cost of synthetic marine salt mixes has facilitated interest in alternative low-cost salt sources and supplementing physiologically important ions. Potassium is involved in ion regulation of intracellular fluids and can be added directly to the water, usually in the form of KCl, or supplemented in the diet (Wilson and El Naggar, 1992).Calcium and magnesium both influence the permeability of osmoregulatory membranes making them critical ions in teleost ionic regulation (Silva *et al.*, 2005). Magnesium is responsible for the activation of numerous enzymes making it a necessary cofactor to the transfer of phosphate groups and the activation of ATP-dependent ion pumps such as NKA (Bijvelds *et al.*, 1998). In freshwater fish, Mg^{2+} deficiency is associated with increased Na^+ and Ca^{2+} levels as well as low K^+ levels (Bijvelds *et al.*, 1997).

Ion regulation is controlled by ionocytes in which there are several proteins involved in ion and water exchange (Evans *et al.*, 2005; Lorin-Nebel *et al.*, 2006). Previous investigations have focused on Na^+/K^+-ATPase (NKA), $Na^+/K^+/2Cl^-$ cotransporter (NKCC), and the cystic fibrosis transmembrane conductance regulator (CFTR) as the three primary proteins involved in ionoregulatory processes (Hirose *et al.*, 2003). The ionoregulatory process is initiated by a basolaterally located NKA which couples two extracellular K^+ with three intracellular Na^- generating an electrochemical gradient that drives the ions according to expression, location, and abundance of NKCC and CFTR (Bodinier *et al.*, 2009; Kang *et al.*, 2008). NKA is responsible for the lowering of intracellular Na^+ concentrations allowing the basolateral NKCC to import Na^+, K^+, and Cl^-, the excess Cl^- is then secreted through the chloride channel CFTR. Additional water exchange and Cl^- ion regulation occurs intestinally (Christensen *et al.*, 2012) and it has also been suggested that substantial Mg^{2+} uptake occurs intestinally (Marshall and Grosell, 2005). The apically located NKCC2 most likely plays an active role in the reabsorption of ions (Lorin-Nebel *et al.*, 2006). The exact mechanism by which Mg^{2+} uptake occurs has yet to be described in detail. It is suggested that significant differences in Mg^{2+} regulation exist among species, knowledge of Mg^{2+} transport across epithelia and cell membranes is still limited (Bijvelds *et al.*, 1998). By exposing euryhaline teleosts to varying salinities or individual ion concentrations, it may be possible to immunolocalize specific osmoregulatory proteins in the gill filaments and intestinal epithelium to better understand the physiological adaptations these species undergo to cope with environmental challenges.

The Gulf killifish, *F. grandis*, inhabits the estuarine waters of the Gulf of Mexico and Atlantic coast of Florida where it is commonly used as a baitfish by anglers for speckled trout, *Cynoscion nebulosus*, flounder, *Paralichthys lethostigma*, and red drum, *Sciaenops ocellatus*. From Texas to Florida it is referred to by many regional names including but not limited to: mudminnow, mudfish, cocahoe minnow, and bull minnow. *F. grandis* are closely related to the mummichog, *F. heteroclitus*, which inhabits the Atlantic coast and is similarly utilized by anglers as a live bait. Both species are characterized as a "hardy" bait tolerant of wide swings in salinity, temperature, and other conditions encountered by live marine bait. *F. grandis* occupy coastal marshes where salinity can change dramatically over a short time period. These fish have the ability to live in salinities that range from freshwater (0 ‰) to near double the concentration of seawater (up to 70 ppt) for several days. Exploitation of this salinity tolerance in *F. grandis* allows investigators the ability to illicit physiological responses across a wide range of ion concentrations.

Several studies have investigated the use of low salinity inland seawater or water from brackish aquifers as a source for marine aquaculture. Much of these inland saline waters were found to have ion deficiencies, where specific manipulation of the major ions in saltwater have significantly increased growth and survival (Doroudi *et al.*, 2006; Fielder *et al.*, 2001; Fotedar *et al.*, 2008). Many studies have investigated the effects of low salinity waters on juvenile and

adult euryhaline species (Coulon *et al.*, 2012; Doroudi *et al.*, 2006; Fielder *et al.*, 2001; Fotedar *et al.*, 2008; Patterson *et al.*, 2012; Roy *et al.*, 2007). Few studies have attempted to explore the importance of specific ion supplementation in euryhaline teleost larviculture. The objectives of this work were to demonstrate the use of physiological examinations at biochemical and molecular levels to determine specific supplementation of monovalent (K^+) and divalent ions (Ca^{2+} and Mg^{2+}) as replacements for saltwater in the culture of *F. grandis* larvae.

Materials and Methods

Three separate four-week experiments were conducted to investigate the impact of external K^+, Ca^{2+} and Mg^{2+} concentrations on larval *F. grandis*. Newly hatched (< 4 h old) *F. grandis* larvae were stocked, in triplicate, at a density of 7 larvae per L into 50-L aquaria maintained on a common recirculating system utilizing biological and ultra violet filters. Larvae were fed a diet of *Artemia sp.* nauplii (30 *Artemia* $fish^{-1}$ d^{-1}) for the first 7 d at a rate of three times per day followed by Otohime™ B1 (Reed Mariculture Inc., Campbell, CA, USA) diet fed to apparent satiation 4 times per day for the remainder of the study. After 4 weeks, survival was calculated by draining all aquaria and counting each individual.

Treatments were established by adding crystal salt (Diamond Crystal Solar Salt, 99.6% NaCl) to 10‰ with ion supplementation.Potassium supplementation consisted of 0.3, 1.3, 2.0and 2.9 mM (Table 1). This investigation represents [K^+] from values similar to

Table 1. Mean salinity and ion concentrations (± SEM) given for each K^+ ,Ca^{2+}, and Mg^{2+} Concentration treatment group. Superscript letters denote statistically significant differences in specific ion concentrations among treatment groups ($P < 0.05$)

	K^+ Treatment group			
Parameter	1	2	3	4**
K^+ (mM)	0.33 ± 0.05^A	1.31 ± 0.04^B	2.06 ± 0.04^C	2.96 ± 0.04^D
Na^+ (mM)	170.19 ± 3.40^A	157.52 ± 6.08^{AB}	151.56 ± 7.03^{AB}	138.01 ± 0.83^B
Mg^{2+} (mM)	0.04 ± 0.00^A	0.12 ± .01^A	0.11 ± 0.01^A	11.55 ± 0.10^B
Ca^{2+} (mM)	0.31 ±0.00^A	0.43 ± 0.05^A	0.30 ± 0.03^A	2.25 ± 0.02^B
Salinity(‰)	9.8 ± 0.28	9.4 ± 0.4	9.4 ± 0.4	9.6 ± 0.1
pH	8.56 ± 0.05	8.27 ± 0.10	8.24 ± 0.09	8.07 ± 0.11
TAN (mg/L)	0.03 ± 0.01	0.02 ± 0.00	0.01 ± 0.00	0.04 ± 0.03
NO_2 (mg/L)	0.00 ± 0.00	0.00 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
Hardness (mg/L)*	37.25 ± 1.55	39.17 ± 2.06	33.83 ± 2.20	1442.67 ± 11.04
Alkalinity (mg/L)*	184.00 ± 25.16	186.00 ± 6.55	182.50 ± 6.98	209.83 ± 3.06

*Reported as $CaCO_3$
** Represents reference salt group

	Ca^{2+} Treatment groups			
Parameter	1	2	3	4
K^+ (mM)	2.37 ± 0.03	2.32 ± 0.02	2.33 ± 0.02	2.31 ± 0.05
Na^+ (mM)	165.31 ± 3.07	166.72 ± 4.83	166.72 ± 4.83	165.84 ± 2.97
Mg^{2+} (mM)	0.11 ± 0.01	0.16 ± 0.02	0.15 ± 0.01	0.12 ± 0.01
Ca^{2+} (mM)	0.20 ± 0.02^A	1.08 ± 0.03^B	1.50 ± 0.04^C	2.07 ± 0.05^D
Salinity(‰)	10.3 ± 0.03	10.1 ± 0.07	10.1 ± 0.06	10.0 ± 0.09
TAN (mg/L)	0.03 ± 0.02	0.03 ± 0.02	0.01 ± 0.01	0.02 ± 0.02
NO_2 (mg/L)	0.05 ± 0.02	0.03 ± 0.02	0.02 ± 0.01	0.01 ± 0.00
Hardness (mg/L)*	193 ± 32.76^A	1090 ± 15.81^B	1433 ± 32.24^C	1740 ± 31.52^D
Alkalinity (mg/L)*	178 ± 17.97	263 ± 57.64	140 ± 4.08	170 ± 7.07

*Reported as $CaCO_3$

	Mg^{2+} Treatment groups			
Parameter	1	2	3	4
K^+ (mM)	2.37 ± 0.03	2.40 ± 0.04	2.29 ± 0.05	2.41 ± 0.02
Na^+ (mM)	168.48 ± 3.64	169.96 ± 4.11	162. 64 ± 4.83	170.55 ± 4.97
Mg^{2+} (mM)	0.05 ± 0.01^A	2.88 ± 0.08^B	5.76 ± 0.16^C	11.52 ± 0.19^D
Ca^{2+} (mM)	0.19 ± 0.02	0.24 ± 0.02	0.15 ± 0.01	0.26 ± 0.02
Salinity(‰)	9.9 ± 0.04	10.2 ± 0.03	10.1 ± 0.03	10.0 ± 0.05
TAN (mg/L)	0.02 ± 0.01	0.01 ± 0.02	0.02 ± 0.02	0.03 ± 0.02
NO_2 (mg/L)	0.01 ± 0.03	0.03 ± 0.02	0.00 ± 0.00	0.02 ± 0.01
Hardness (mg/L)*	102 ± 25.82^A	320 ± 44.82^B	530 ± 29.35^C	1040 ± 36.21^D
Alkalinity (mg/L)*	165 ± 19.53	160 ± 4.71	150 ± 10. 62	185 ± 21.83

*Reported as $CaCO_3$

freshwater sources (0.33 mM) to concentrations of 2.96 mM, found saline waters at 10 ‰. Potassium concentration in [Ca^{2+}] and [Mg^{2+}] experiments were maintained at 2.35 mM, as determined by a previous study (Fisher *et al.*, 2013). Calcium was supplemented to give final concentrations of 0.20, 1.08, 1.50, and 2.07 mM (Table 1), while Mg^{2+} was supplemented to give concentrations of 0.05, 2.88, 5.76, and 11.52 mM. The [Mg^{2+}] experiment also received Ca^{2+} supplementation to the water in the form of $CaCO_2$, as determined in the previous experiment (Table 1). Weekly water samples were collected to quantify ion concentrations via ICP analysis by the LSU Agriculture Center; Agriculture Chemistry Department (Baton Rouge, LA, USA).

Fish were sampled at 0, 1, 3, 7, 10, 14, and 28 d post hatch (DPH). Upon each sampling the standpipe was adjusted to maintain a constant density for each treatment. Three replicates of 5 larvae were used to establish an average desiccated dry mass due to the inability to accurately measure individual dry mass of larvae less than 7 dph. Whole body ion concentration (Na^+, K^+ and Mg^{2+}) was analyzed using the methods described by (Van Genderen, 2003; Fisher *et al.*, 2013). Three replicates of 5 larvae were collected at each time point to determine Na^+/K^+-ATPase activityusing the protocol described in McCormick (1993) and expressed as μmol ADP mg protein^{-1} h^{-1}.

Six larvae were collected per treatment group at 1, 3, and 7 dph and preserved in Z-fix (Anatech, LTD, Battle Creek, MI, USA). Immunocytochemistry reactions were performed according to methods described in Bodinier *et al.* (2010) and Fisher *et al.* (2013) for NKA, CFTR, and NKCC1. Ionocyte length measurements were taken using Image Tool® version 3.0 (University of Texas Health Science Center, San Antonio, TX, USA). A minimum of 50 cells were measured per sampling period for each treatment.

Results

Growth and survival: The 0.3 mM [K^+] treatment resulted in 100% mortality within 24 h of hatch. No significant differences were seen in survival between the 1.3 and 2.0 mM [K^+] groups (survival ≤ 5%); however the 2.9 mM [K^+] treatment resulted in significantly greater survival after 4 weeks (survival ~60%). Final dry mass of the 2.9 mM [K^+] reference group was significantly higher than both the 1.3 and 2.0 mM [K^+] treatments ($P \leq 0.05$). The 2.9 mM [K^+] reference group had a final dry mass of 4.93 ± 0.54 mg while the 1.3 and 2.0 mM [K^+] treatments had dry mass of 2.39 ± 0.19 mg and 2.21 ± 1.55 mg, respectively.

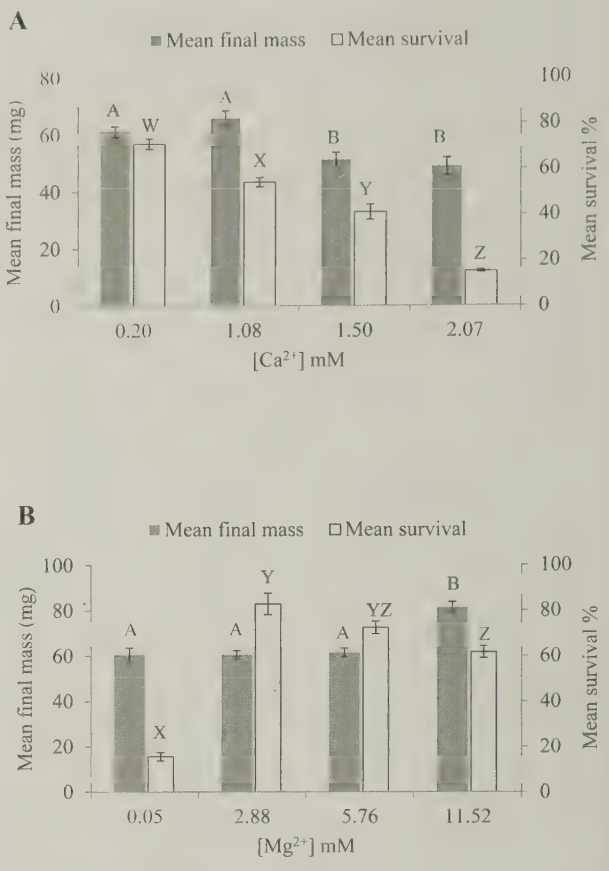


Fig. 1. Final mass and survival of Gulf killifish larvae after 4 weeks in (A) [Ca^{2+}] treatments or (B) [Mg^{2+}] treatments. Letters represent significant differences among treatment groups. Letters denote significant differences among treatments.

Survival and dry mass were significantly influenced by both [Ca^{2+}] and [Mg^{2+}] experiments. Supplementation of Ca^{2+} showed a decrease in mean final mass and mean survival with increasing concentrations of Ca^{2+} in the absence of Mg^{2+} (Fig. 1A). Supplementation of Mg^{2+} resulted in approximately a 5-fold increase in survival with Mg^{2+} supplementation as compared to no supplementation, no differences were observed among supplemented treatment groups. Final mass was not significantly

influenced until a concentration of 11.52 mM Mg^{2+} was reached (Fig. 1B).

Whole-body ion concentrations & Na^+/K^+-ATPase activity: No differences were found in Na^+/K^+-ATPase activity among $[K^+]$ treatment groups or among time ($P \geq 0.05$). Whole body Mg^{2+} concentration showed no difference for treatment or treatment × dph interaction; however significant difference was determined for dph ($P \leq 0.05$). There was a significant increase in $[Mg^{2+}]$ within the first week followed by a rapid decrease for all three$[K^+]$ treatment groups. Whole body K^+ concentration had no significant difference in treatment × dph interaction, although significant differences were observed among treatments and for dph ($P \leq 0.05$).

No differences were observed among treatments for whole body ion composition in the $[Ca^{2+}]$ study. The $[Mg^{2+}]$ experiment showed delayed peaks in whole body $[Na^+]$, $[Ca^{2+}]$, and $[K^+]$ for the 0.05 mM $[Mg^{2+}]$ treatment group, with ion concentrations reaching their highest point 7 days later than other treatments. As expected, the Mg^{2+} deficient treatment groups (0.05 and 2.70 mM $[Mg^{2+}]$) also showed a significant decrease in whole body $[Mg^{2+}]$.

Na^+/K^+ -ATPase activity in the $[Ca^{2+}]$ study was significantly lower at 0 and 1 dph followed by a significant increase in activity throughout the experiment, however no treatment specific differences in activity were determined at any specific sample dates. The $[Mg^{2+}]$ study demonstrated significant differences in Na^+/K^+ -ATPase activity between Mg^{2+} deficient treatments and those with sufficient supply at 0 dph followed by minor, but not significant, increases throughout the experiment (Fig. 2).

Immunocytochemistry & cell volume: All negative slides, with no primary antibody, showed no immunostaining as expected (not illustrated). In $[K^+]$ treatments at 7 dph, no significant differences were determined among treatments for gill ionocyte length. Gill ionocytes from the 1.3 mM $[K^+]$ treatment were found to have a significantly larger area than those in the 2.0 and 2.9 mM $[K^+]$ treatments. Intestinal CFTR and NKA were present at 7 dph in all surviving $[K^+]$ treatment groups. No changes were observed in NKA or CFTR in the gill ionocytes. Intestinal NKA and CFTR staining appeared to show a decrease in intensity as K^+ concentration increased (Fig. 3). Very little staining was observed for NKCC both intestinally and at the gill epithelium.

No differences among treatments were observed

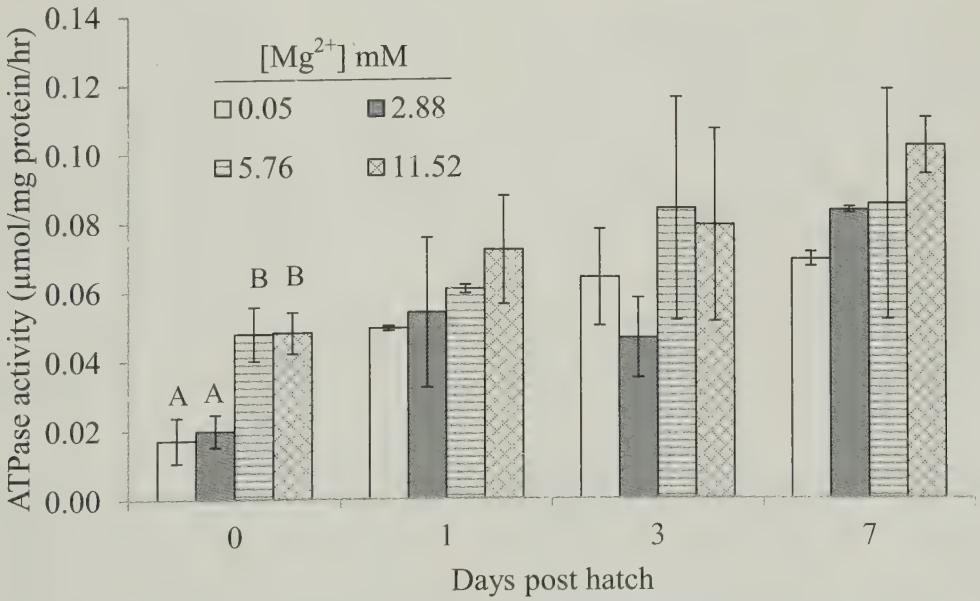


Fig. 2. Whole body NKA activity for larvae stocked in $[Mg^{2+}]$ treatments after 0, 1, 3, and 7 dph. Letters represent significant differences among treatment groups at that day.

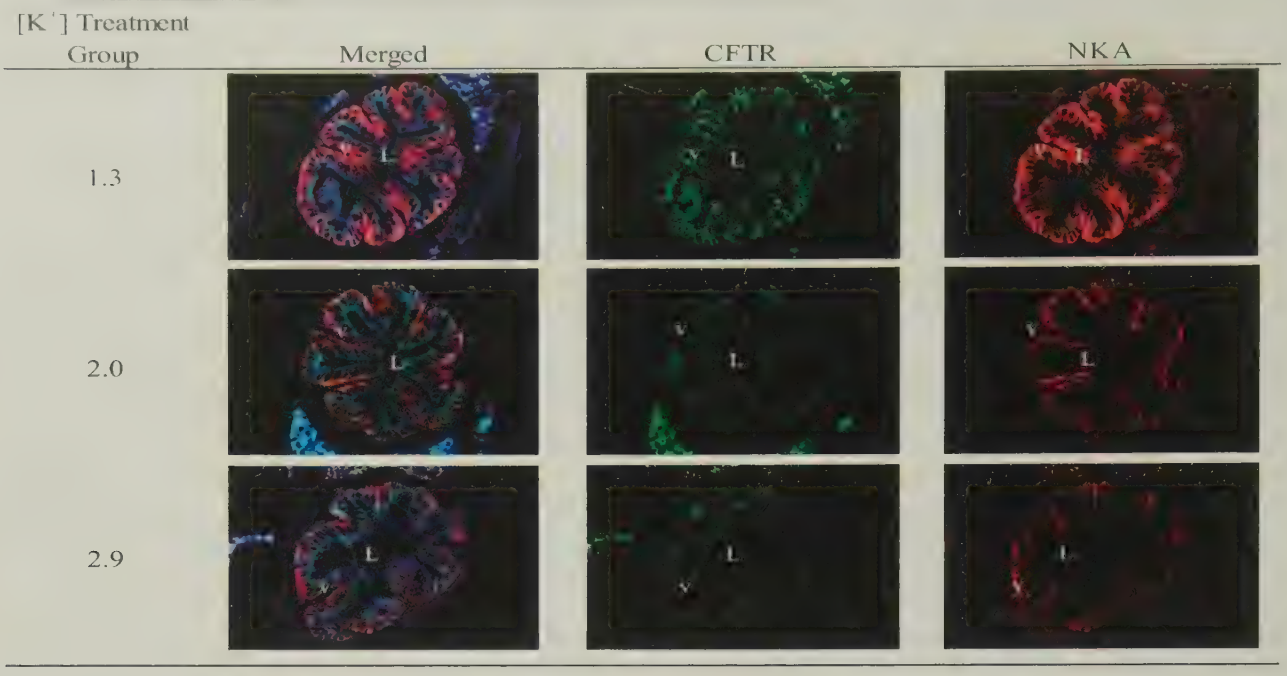


Fig. 3. Representative histological sections from larval intestines after one week in [K^+] treatment groups cut transversely at 5 μm intervals. Immunofluorescence of NKA (red) and CFTR (green). The 2.9 mmol K^+ group represents a reference salt used andcontains other ions which may be essential for the function of osmoregulatory proteins. L: lumen; v: villi

for NKA or NKCC staining intensity in the gills or intestine, nor were there any significant differences among treatments for gill ionocyte length or quantity at 1, 3, and 7 dph for both the [Ca^{2+}] and [Mg^{2+}] experiments. In the [Mg^{2+}] experiment, a decrease in CFTR staining intensity was observed at 1 dph in both the intestine (Fig. 4) and gill in the 0.05 mM [Mg^{2+}] treatment, no other changes in CFTR staining intensity were observed.

Discussion

The chemical gradient generated by the activation of NKA is one of the primary driving forces behind ion regulation, thus the ability to measure whole body NKA activity may allow for the evaluation of larval fish osmoregulation capacity in hyper and hypo-osmotic environments. It is possible that K^+ is a limiting factor in killifish larval osmoregulation and a deficiency can restrict the ability of NKA to function. The current model for chloride secretion as described by McCormick *et al.* (2003) suggests a basolateral NKA and NKCC coupled with an apically located CFTR-like protein. Because K^+ is also used by the chloride cotransporter, NKCC, to assist in the transport of chloride ions, an extracellular K^+ shortage could reduce a larval fish's ability to regulate sodium and chloride ion concentrations at critical biological membranes. Despite the fact that there were no statistical differences in NKA activity between any treatment groups, it is still conceivable that sufficient K^+ was not available for the function of NKCC. If so then the inability of the larvae to regulate either Na^+ or Cl^- ions may possibly have been a factor in the low survival of the 0.3, 1.3 and 2.0 mM [K^+] groups. The reference group (marine mix salt) was the only group that did not display poor survival and it is possible that the presence of other essential ions such as Mg^{2+} and Ca^{2+} played a significant role in the survival of this treatment group.

Potassium supplementation across the gradient examined in the current study at a constant salinity not only altered survival, but affected differences among molecular components of intestinal epithelium. Although potassium treatments in the current study did not appear to alter whole body Na/K ATPase activity, immunofluorescence

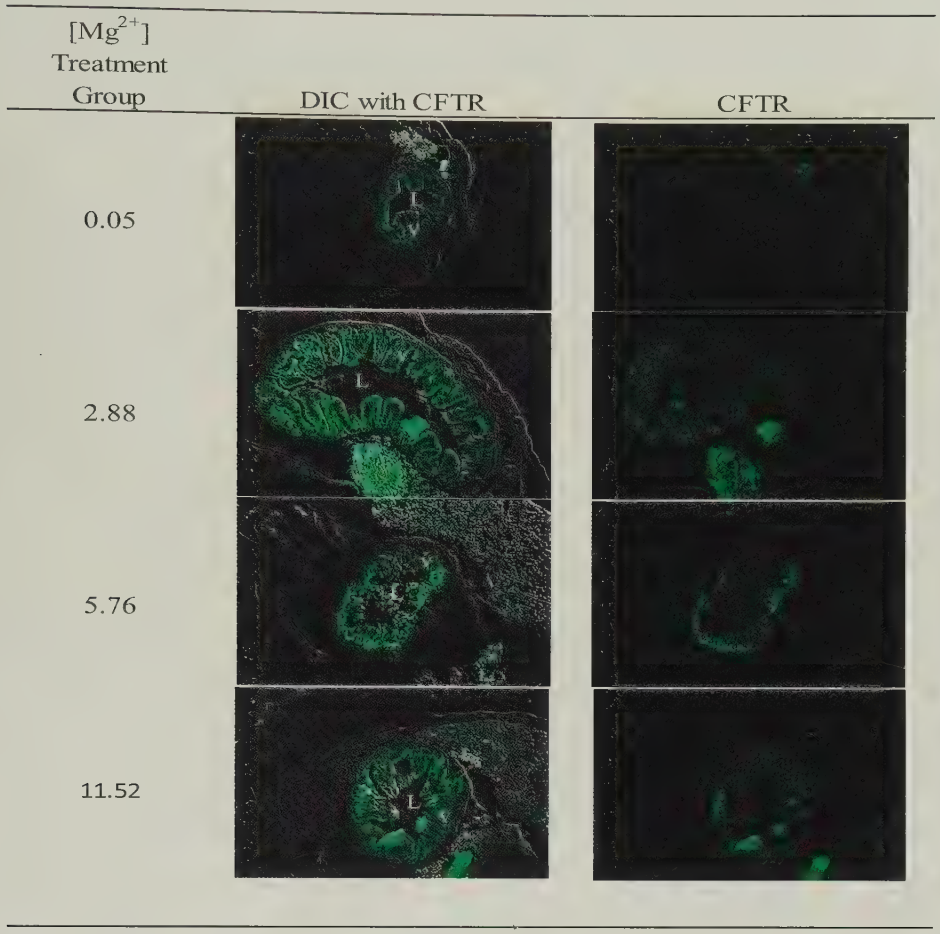


Fig. 4. Representative histological sections of the intestine from 1 day post hatch (dph) Gulf killifish larvae in [Mg^{2+}] treatment groups, cut transversely at 5 μm intervals. Differentialinterference contrast (DIC) images with immunofluorescence of CFTRL: lumen; v: vill

showed increased density of NKA and CFTR proteins as K^+ concentration decreased. Whole body K^+ concentration was greater in the reference as compared to treatments with 1.3 and 2.0 mM of K^+ both of which remained lower throughout the study as expected due to the inability to selectively retain K^+ ions. It is important to note that the reference group containing the highest concentration of K^+ was also comprised of greater concentrations of Mg^{2+} and Ca^{2+}, which could have modulated Na^+ and Cl^- absorption in the intestine relative to lower K^+ treatments in the current study that were relatively low in these divalent ions (Marshall and Singer, 2002; O'Grady, 1989). Growth and survival metrics for the reference treatment may reflect combined effects of a full to partial complement of other ions in addition to the K^+ gradient examined. The role of intestinal epithelium in ion uptake not only allows the potential for uptake and maintenance of ion balance from dietary sources and thus important in aquaculture, but also demonstrates the potential to modulate concentrations of specific ions in prepared waters for euryhaline and marine teleosts.

Calcium and Magnesium are critical in teleost ion regulation, varying concentrations of Ca^{2+} and Mg^{2+} can impact the permeability of osmoregulatory epithelia to both ions and water (Silva *et al.*, 2005). In the sea water adapted eel, removal of Ca^{2+} from the external medium diminished the active excretion of sodium by half, excretion returned to normal level after the reintroduction of Ca^{2+} to the medium (Isaia and Masoni, 1976). Proper development of many euryhaline teleosts is related to environmental Ca^{2+} concentration, for example red drum (*Sciaenops ocellatus*) fry displayed a drop in blood osmolarity when transferred from salt to freshwater, this

decline in osmolarity was reduced by the addition of Ca^{2+}(Wurts and Stickney, 1989). Previous studies have determined that Mg^{2+} is critical in the activation of ATP-dependent ion pumps (Bijvelds *et al.*, 1998) making it critical for the activation of NKA. In this study, both the 0.05 and 2.88 mM [Mg^{2+}] treatment groups displayed significantly reduced NKA activity at hatch.

In the current study both Ca^{2+} and Mg^{2+} had effects on survival, growth, and molecular components at both the gill filaments and intestinal epithelium. In the absence of Mg^{2+} supplementation larval *F. grandis* had reduced survival, decreased Na^{+}/K^{+} -ATPase activity, and a delayed peak in whole body compositions for several ions essential for larval development. A decrease in CFTR intensity was also observed in the gills and intestine of Mg^{2+} deficient treatment as seen by immunocytochemistry staining at 1 dph. This is an indication that Mg^{2+} is a necessity for larval *F. grandis* growth, survival, and ion regulation. However, the Mg^{2+} of 10‰ seawater is approximately 11.52 mM and the greatest growth and survival were achieved in this study at a concentration of 2.88 mM Mg^{2+}. These results demonstrate that the potential for euryhaline teleost culture in prepared water may only require a fraction of the ion concentrations found in 10‰ seawater. Also the role of intestinal epithelium in ion uptake allows for the potential for ion maintenance through dietary sources.

Acknowledgements

This work was supported, in part, through grants from the Southern Regional Aquaculture Center and Louisiana SeaGrant. The authors would like to acknowledge the faculty, students and support staff that assisted with these investigations: Paige O'Malley, Emily McArdle, Chris Mariani, Mike Coulon, Josh Patterson, Charlie Brown.

References

Bijvelds M. J. C., Flik G., and Bonga S. E. W., 1997: Mineral balance in Oreochromis mossambicus: dependence on magnesium in diet and water. *Fish Physiol. Biochem.*, **16**, 323-331.

Bijvelds M., Velden J., Kolar Z., and Flik G., 1998: Magnesium transport in freshwater teleosts. *J. Exp. Biol.*, **201**, 1981-1990.

Bodinier C., Boulo V., Lorin-Nebel C., and Charmantier G., 2009: Influence of salinity on the localization and expression of the CFTR chloride channel in the ionocytes of *Dicentrarchus labrax* during ontogeny. *J. Anat.*, **214**, 318-329.

Bodinier C., Sucré E., Lecurieux-Belfond L., Blondeau-Bidet E., and Charmantier G., 2010: Ontogeny of osmoregulation and salinity tolerance in the gilthead sea bream Sparus aurata. *Comp. Biochem. Physiol. - Part A: Mol. Integrat. Physiol.*, **157**, 220-228.

Christensen A. K., Hiroi J., Schultz E. T., and McCormick S. D., 2012: Branchial ionocyte organization and ion-transport protein expression in juvenile alewives acclimated to freshwater or seawater. *J. Exp. Biol.*, **215**, 642-652.

Coulon M., Gothreaux C., and Green C., 2012: Influence of substrate and salinity on air-incubated Gulf killifish embryos. *North American J.Aquacult.*, **74**, 54-59.

Doroudi M. S., Fielder S D., Allan G L., and Webster G. K., 2006: Combined effects of salinity and potassium concentration on juvenile mulloway (*Argyrosomus japonicus*, Temminck and Schlegel) in inland saline groundwater. *Aquacult. Res.*, **37**, 1034-1039.

Evans D. H., Piermarini P. M., and Choe K. P., 2005: The multifunctional fish gill: Dominant site of gas exchange, osmoregulation, acid-base regulation, and excretion of nitrogenous waste. *Physiological Rev.*, **85**, 97-177.

Fielder S. D., Bardsley W. J., and Allan G. L., 2001: Survival and growth of Australian snapper, *Pagrus auratus*, in saline groundwater from inland New South Wales, Australia. *Aquacult.*, **201**, 73-90.

Fisher C., Bodinier C., Kuhl A., and Green C., 2013: Effects of potassium ion supplementation on survival and ion regulation in Gulf killifish *Fundulus grandis* larvae reared in ion deficient saline waters. *Comp. Biochem. Physiol. Part A: Mol Integrat. Physiol.*, **164**, 572-578.

Fotedar R., Harries S., and Savage S., 2008: Survival,

growth and osmolality of greenlip abalone *Haliotis laevigata* (Donovan 1808) when exposed to different ionic profiles of inland saline water. *Aquacult. Res.*, **39**, 441-448.

Hirose S., KanekoT., Naito N., and Takei Y., 2003: Molecular biology of major components of chloride cells. *Comp. Biochem. Physiol. Part B: Biochem. Mol. Biol.*, **136**, 593-620.

Isaia J., and Masoni A., 1976: The effects of calcium and magnesium on water and ionic permeabilities in the sea water adapted eel, *Anguilla anguilla* L. *J. Comp. Physiol. B*, **109**, 221-233.

Kang C. K., Tsai S. C., Lee T. H., and Hwang P. P., 2008: Differential expression of branchial Na+/K+-ATPase of two medaka species, Oryzias latipes and Oryzias dancena, with different salinity tolerances acclimated to fresh water, brackish water and seawater. *Comp. Biochem. Physiol. - Part A: Mol. Integrative Physiol.*, **151**, 566-575.

Lorin Nebel C., Boulo V., Bodinier C., and Charmantier G., 2006: The Na+/K+/2Cl- cotransporter in the sea bass *Dicentrarchus labrax* during ontogeny: involvement in osmoregulation. *J. Exp. Biol.*, **209**, 4908-4922.

Marshall W. S., and Grosell M., 2005: Ion transport, osmoregulation, and acid-base balance, *Fish Physiol.*, 177-230.

Marshall W. S., and Singer T. D., 2002: Cystic fibrosis transmembrane conductance regulator in teleost fish. *Biochim. Biophys. Acta-Biomembr.*, **1566**, 16-27.

McCormick S. D., 1993: Methods for nonlethal gill biopsy and measurement of Na+, K+-ATPase activity. *Can. J. Fisheries Aquat. Sci.*, **50**, 656-658.

McCormick S. D., Sundell K., Bjornsson B. T., Brown C. L., and Hiroi J., 2003: Influence of salinity on the localization of Na^+/K^+-ATPase, $Na^+/K^+/2Cl^-$ cotransporter (NKCC) and CFTR anion channel in chloride cells of the Hawaiian goby (*Stenogobius hawaiiensis*). *J. Exp. Biol.*, **206**, 4575-4583.

O'Grady S. M., 1989: Cyclic nucleotide-mediated effects of ANF and VIP on flounder intestinal ion transport. *Am. J. Physiol.-Cell Physiol.*, **256**, C142-C146.

Patterson J., Bodinier C., and Green C., 2012: Effects of low salinity media on growth, condition, and gill ion transporter expression in juvenile Gulf killifish, *Fundulus grandis*. Comp. Biochem. Physiol. - Part A: Mol. Integrative Physiol., **161**, 415-421.

Roy L. A., Davis D. A., SaoudI P., and Henry R. P., 2007: Supplementation of potassium, magnesium and sodium chloride in practical diets for the Pacific white shrimp, *Litopenaeus vannamei*, reared in low salinity waters. *Aquacult. Nutrition*, **13**, 104-113.

Silva L. V. F. D., Golombieski J. I., and Baldisserotto B., 2005: Growth and survival of silver catfish larvae, Rhamdia quelen (Heptapteridae), at different calcium and magnesium concentrations. *Neotropical Ichthyology*, **3**, 299-304.

Van Genderen E. J., 2003: Influence of soft surface water conditions on copper toxicity to *Pimephales promelas* Rafinesque: responses at mechanistic-, population-, and watershed- levels of organization. Clemson University, Clemson, SC, USA.

Wilson R. P., and El Naggar G., 1992: Potassium requirement of fingerling channel catfish, *Ictalurus punctatus*. Aquacult., **108**, 169-175.

Wurts W. A., and Stickney R. R., 1989: Responses of Red drum (*Sciaenops ocellatus*) to calcium and magnesium concentrations in fresh and salt water. Aquacult., **76**, 21-35.

Annotated Bibliography

Fisher C., Bodinier C., Kuhl A., and Green C., 2013: Effects of potassium ion supplementation on survival and ion regulation in Gulf killifish *Fundulus grandis* larvae reared in ion deficient saline waters. *Comp. Biochem. Physiol., Part A.*, **164**, 572-578.

This study encompasses results pertaining to the K^+ ion manipulation portions of the abstract presented above for the UJNR Scientific Symposium. This investigation increased $[K^+]$ from values similar to freshwater sources (0.33 mM) to concentrations of 2.96 mM, found in saline waters at 10‰. A number of biochemical and molecular techniques

were performed to examine the effect of this K^+ ion gradient, which included: whole body ion composition, Na^+/K^+ -ATPase (NKA) activity, gill ionocyte morphometrics, relative gene expression (NKA, NKCC, and CFTR), and corresponding immunocytochemistry at the gill and intestinal epithelium. Results more tangible to aquaculturist, such as growth and survival, indicated the presence of a threshold within the gradient examined whereby survival was not different between 1.3 to 2.9 mM $[K^+]$. Utilizing immunocytochemistry, the differences between these seemingly similar treatment groups indicated that the treatments groups between 1.3 to 2.9 mM $[K^+]$ was different in terms of gill ionocyte area, NKA and CTFR localization.

Ostrowski A. D., Watanabe W. O., Montgomery F. P., Rezek T. C., Shafer T. H., Morris Jr J. A., 2011: Effects of salinity and temperature on the growth, survival, whole body osmolality, and expression of Na+/K+ ATPase mRNA in red porgy (*Pagrus pagrus*) larvae. *Aquacult.*, **314**, 193-201.

These authors investigated a number of physiological parameters pertaining to salinity and temperature in embryos and larvae of red porgy (*Pagrus pagrus*), which is viewed as a high-market value marine species with good potential as an aquaculture species. Embryos and resulting larvae were reared at four temperatures (17, 19, 21, 23℃) and two salinities (24 and 34‰). Larvae (16 dph) were transferred from their respective salinities to 44‰ to represent a sublethal hyperosmotic challenge. These authors demonstrated significant increases in NKA mRNA expression in individuals acclimated to 24‰ at 24 h after transferred to increased salinity, while individuals from 34‰ exhibited no significant changes in NKA expression. Temperature was not observed to influence expression of NKA, while metabolic parameters related to growth were influenced by the temperature gradient in their study. The authors utilized traditional growth metrics including molecular tools to anticipate optimum salinity (24‰) and temperature (23℃) conditions that are optimum for larval rearing of this species.

Bodinier C., Sucré E., Lecurieux-Belfond L., Blondeau-Bidet E., Charmantier G., 2010: Ontogeny of osmoregulation and salinity tolerance in the gilthead sea bream Sparus aurata. Comp. Biochem. Physiol. - Part A: Mol. Integrative Physiol., **157**, 220-228.

The gilthead sea bream (*Sparus aurata*) is a commercially important aquaculture species, which spawns in the ocean and whose resulting larvae and juveniles migrate to lower salinity estuaries and lagoons. These authors investigated the development of salinity tolerance in gilthead sea bream from 3, 30, 75, 96, and 300 d post hatch (dph) by challenging them with 9 salinities ranging between freshwater and 45.1‰. Utilizing immunohistochemistry, these authors localized the NKA throughout these challenges to document location of ion regulation within respect to this ionoregulatory protein. Initially, immunopositive NKA ionocytes were laocted in the integument along the yolk sac and integumentary folds representing the branchial slits. A functional shift from integument to gills was demonstrated 30 and 70 dph, when both the integument and gills were observed to locally express NKA, whereby from 70 to 300 dph the gills remain the main site of osmoregulation. Increases in osmoregulatory capacity for this species at the intervals examined within this study related to the shifts and patterns observed through immunohistochemistry.

Christensen A. K., Hiroi J., Schultz E. T., McCormick S. D., 2012: Branchial ionocyte organization and ion-transport protein expression in juvenile alewives acclimated to freshwater or seawater. *J. Exp. Biol.*, **215**, 642-652.

In gill ionocytes, ion transport is activated by the basolaterally located NKA which generates an electrochemical gradient by coupling two extracellular K^+ with three intracellular Na^+, driving the ions according to expression, location, and abundance of other proteins such as NKCC and CFTR. Christensen *et al.* investigated changes in alewife physiology and branchial epithelium as individuals were acclimated to freshwater or saltwater and represents the first study of its kind to characterize multiple ion-transport proteins in a non-salmonid anadromous fish. Corresponding

increases in NKA, NKCC1, and CFTR abundance at the gill epithelium with increasing salinity was used to establish a gill model for hypo-osmoregulation. In gill ionocytes, NKA is responsible for lowering intracellular Na^+ allowing the basolateral NKCC1 to import Na^+, K^+, and two Cl^- ions. Excess intracellular Cl^- is then secreted through the CFTR chloride channel. NKCC1, the secretory isoform is expressed basolaterally in the gill ionocytes, while NKCC2 is identified as an absorptive isoform and is expressed apically along the intestinal and urinary bladder epithelium of saltwater and euryhaline teleosts. The authors determined the key differences between freshwater and seawater acclimated alewives in the context of ion transporters at the gill epithelium. The implications of these investigation has assisted in increasing the information multicellular complexes of mature ionocytes and the role of salinity and specific ion in the maintenance of homeostasis.

水研センター研報，第40号，109－110，平成27年
Bull. Fish. Res. Agen. No. 40, 109－110, 2015

Genetically Modified Salmon in Aquaculture: Well Regulated and Safe

Paul G. Olin*[1]

Abstract: A Massachusetts company, Aquabounty Technologies, submitted an application to the U.S. Food and Drug Administration (FDA) in 1995 to grow a genetically modified AquAdvantage® Atlantic salmon to be marketed as a food product. Aquabounty proposed to raise the broodstock fish on Prince Edward Island, ship their eggs to a contained inland recirculating production system in Panama to grow, harvest and process the fish, and then ship food grade product back to the United States for sale. The fish for this physically secure production system would be at least 99 percent triploid and all-female, as an additional reproductive-containment measure.

The AquAdvantage® Atlantic salmon carries a Chinook salmon growth hormone gene that results in production of growth hormones that enable the fish to grow to market weight in half the time it normally takes (Fig. 1) This gene is regulated by a segment of DNA from the ocean pout, a blenny-like fish found in frigid waters of the Northwest Atlantic.

The review process used by FDA on the AquAdvantage® salmon involved a team of scientists and subject-matter experts on the Veterinary Medicine Advisory Committee who advise the FDA and the general public on scientific issues as they relate to ensuring public and animal health. The FDA released a 172 page briefing packet and an 84 page environmental assessment containing information relevant to the application in advance of a public advisory committee meeting held in September 2010. This briefing packet summarized their scientific review, and the basic conclusions were that the AquAdvantage® salmon are safe, nutritionally comparable to other Atlantic salmon, and when produced as described in the application do not pose a threat to the environment. The review process used by FDA on the AquAdvantage® salmon was rigorous, detailed and extensive, spanning more than 15 years. Specific conclusions from the report stated that "Food from AquAdvantage® Salmon is the same and as safe to eat as food from other Atlantic salmon."

Fig. 1. AquAdvantage® and non-GM Atlantic salmon of similar age.

In reference to concerns about adverse environmental impacts the report states "There is substantial, reliable information available in the environmental assessment document to conclude that AquAdvantage® Atlantic salmon are not expected to have a significant impact on the environment when raised and reared under the current conditions of physical, biological and geographical/geophysical confinement present at hatchery and grow-out facilities in Canada and Panama. We have a high degree of certainty in our conclusions regarding AquAdvantage® Salmon."

Despite this rigorous scientific review, there remains considerable public controversy regarding the potential for FDA approval of this Aquabounty application to produce and market a genetically

2015年1月30日受理 (Received on January 30, 2015)
*1 UC San Diego, Scripps Institution of Oceanography, La Jolla, CA 92093-0232
E-mail: polin@ucsd.edu

modified salmon. As a result, 11 senators signed a request that FDA stop the process for approving genetically modified AquAdvantage® Atlantic salmon. The manner in which this review for approval of a transgenic animal for agricultural production and marketing in the United States has progressed has stymied American research in this promising sector of animal biotechnology.

New technologies to genetically improve food and animal crops are one tool to supply the additional food people will need in the future, improving human health, reducing the use of pesticides and fertilizers, and lessening the carbon footprint of animal and plant agriculture. A 2008 scientific review published in the Journal of the Royal Society of Medicine noted that genetically modified foods had been eaten by millions of people worldwide for 15 years, with no reports of ill effects.

Annotated Bibliography

Van Eenennaam, Alison L., and Muir William M., 2011: Transgenic salmon: a final leap to the grocery shelf? Nature Biotechnology, **29**, 706–710.

This paper presents a detailed analysis of the regulatory and review process that FDA used for the AquAdvantage® salmon.

Entis, Elliot and Greenberg, Paul. 2013: The Salmon Dialogue: What's the future of sustainable, genetically engineered foods? Genetic Literacy Project.http://www.geneticliteracyproject.org/2013/09/17/the-salmon-dialogue-whats-the-future-of-sustainable-genetically-engineered-foods/#.UjpTgtLBOSo

This online debate features, Elliot Entis, whose company has created a genetically modified salmon that may soon be for sale in the U.S., who discusses the environmental and health impacts of this controversial technology with author Paul Greenberg, a critic of GM fish.

Menozzi, Davide, Mora, Cristina and Merigo, Alberto. 2012. Journal of Agrobiotechnology Management & Economics, Volume 15, Number 3.

In this paper the authors provide an overview of salmon farming and world markets and then analyze three scenarios based on the level of acceptance of GM salmon in the marketplace. The three scenarios were named as 1) 'no market for GM fish,' 2) 'GM salmon for dinner,' and 3) 'GM salmon doesn't take off.' The authors provide a summary of the main outcomes for each scenario.

FDA 2012.AquAdvantage® Salmon, Draft Environmental Assessment.Center for Veterinary Medicine, United States Food and Drug Administration, Department of Health and Human Services. Washington D.C.

This document provides a detailed environmental assessment of the transgenic AquAdvantage® Salmon

水研センター研報，第40号，111－119，平成27年
Bull. Fish. Res. Agen. No. 40, 111－119, 2015

Reproductive Dysfunction in Cultured Sablefish (*Anoplopoma fimbria*)

José M. GUZMÁN[*1], J. Adam LUCKENBACH[*1,2], Frederick W. GOETZ[*1], William T. FAIRGRIEVE[*1], Mollie A. MIDDLETON[*3], and Penny SWANSON[*1,2]

Abstract: Sablefish *Anoplopoma fimbria* is a ground fish native to the North Pacific Ocean that is considered a promising new species for marine aquaculture in the US. However, efforts to establish sustainable production of sablefish have been constrained by the reproductive performance of females from the first-filial (F1) generation. Although some F1 females may mature after 5+years of age, some others fail to initiate puberty in captivity. Development of methods to unblock/induce early puberty are necessary to reduce costs associated with rearing F1 female broodstock and reduce generation times for selective breeding.

Current research at the Northwest Fisheries Science Center (NOAA, Seattle, USA) integrates basic and applied biology to gain knowledge on the reproductive endocrine system of sablefish and develop approaches to unblock or reduce the age of puberty in F1 female sablefish. As part of our basic line of research, we compared the pituitary gonadotropin-ovary axis in wild-caught, maturing females and 8 year-old F1 females that had never shown signs of sexual maturation. Wild-maturing females had higher levels of pituitary gonadotropin subunit and ovarian gonadotropin receptor mRNAs and plasma sex steroids compared to F1 females, which were holding at the immature, perinucleolus ovarian stage. Anecdotal evidence from sablefish farms indicates that F1 female broodstock maintained in ~4 ℃ seawater mature in captivity. We hypothesize that culture conditions that use warmer water (10–15 ℃) suppress the pituitary gonadotropin-ovary axis in sablefish, and ultimately block the onset of puberty.

As part of our applied line of research, we conducted a series of studies to determine the ability of exogenous hormones to stimulate the reproductive axis in prepubertal F1 females. Treatments with testosterone or estradiol 17-beta (E2) increased the expression of pituitary luteinizing hormone beta subunit 40- and 185-fold, respectively, relative to control. This finding suggests that pituitaries from immature females are responsive to exogenous hormones, and that sex steroids may be an important part of a hormone therapy to stimulate the reproductive axis of F1 females. In addition, using an *in vitro* ovarian tissue culture system, we demonstrated that fragments of prepubertal sablefish ovaries incubated with human-chorionic gonadotropin increased the secretion of E2. This indicates that ovaries of prepubertal females are equipped to synthesize and release sex steroids critical for vitellogenesis under the appropriate hormone stimulation, and that the failure to initiate puberty is likely due to a lack of adequate gonadotropin signaling. These data provide the foundation for the development of hormone treatments aimed at inducing puberty in prepubertal F1 female sablefish.

Key words: sablefish, marine aquaculture, reproduction, gonadotropins

2015年1月30日受理（Received on January 30, 2015）
*1 Environmental and Fisheries Sciences Division, Northwest Fisheries Science Center, National Marine Fisheries Service, National Oceanic and Atmospheric Administration, Seattle, WA 98112, USA
*2 Center for Reproductive Biology, Washington State University, Pullman, WA 98164, USA
*3 School of Aquatic and Fishery Sciences, University of Washington, Seattle, WA
Corresponding author: José M. GUZMÁN, E-mail: jose.guzman-jimenez@noaa.gov

In fishes, as in other vertebrates, reproduction is primarily controlled by the hypothalamic-pituitary-gonadal axis. The hypothalamic neuroendocrine system regulates synthesis and release of the pituitary gonadotropins, follicle-stimulating hormone (Fsh) and luteinizing hormone (Lh). Both gonadotropins are essential in the endocrine control of reproduction by regulating steroidogenesis and germ cell development through interactions with their respective receptors in gonadal tissues (Levavi-Sivan *et al.*, 2010; Zohar *et al.*, 2010).

Optimal function of the reproductive endocrine system is critical for fish to complete gonadal development, maturation and spawn successfully. Under culture conditions, however, many fish species exhibit some degree of reproductive dysfunction. These can vary from inconsistent spawning to a complete failure to undergo puberty (i.e., the time during which an individual becomes capable of reproducing for the first time) (Mylonas *et al.*, 2010; Taranger *et al.*, 2010). Such a delay in age of puberty poses a major problem for selective breeding in finfish aquaculture and necessitates further research.

Hormone therapies are widely used to control reproduction in cultured fish species. During the past two decades, hormone therapies have been developed to induce puberty in striped bass (*Morone saxatilis*) (Holland *et al.*, 2002), European sea bass (*Dicentrarchus labrax*) (Zanuy *et al.*, 1999), European eel (*Anguilla anguilla*) (Vidal *et al.*, 2004), and grey mullet (*Mugil cephalus*) (Aizen *et al.*, 2005). Therapies for inducing puberty or to accelerate this process were based on an understanding of how the endogenous reproductive system is hormonally regulated and where the insufficiency/failure occurs in the reproductive system (e.g., brain, pituitary gland and/or gonad) that delays or blocks puberty onset. Therefore, characterization of the reproductive dysfunction in a given species helps to tailor specific strategies to optimize its reproduction in captivity.

Sablefish (*Anoplopoma fimbria*), known as *gindara* in Japan, is a groundfish native to the North Pacific Ocean ranging from Baja California to Alaska's Bering Sea and Japan. The primary market for sablefish is in Japan, where demand and prices are high, but an increasing amount of the wild catch is staying in the US market. Due to its rapid growth rate (1.5 kg after 12-months of growout) and high market value (22 USD/lb. approx. market price), sablefish has been identified as an excellent marine aquaculture species in the US. However, efforts to establish sustainable and efficient production have been constrained by the reproductive performance of females from the first filial (F1) generation (i.e., produced and maintained in captivity). Although some F1 females may mature at 5+ years old in the industry setting, others never initiate puberty in captivity (B. Campbell, Sablefish Canada Inc., personal communication). This situation compromises the development of selective breeding programs and increases costs associated with rearing female sablefish broodstock.

Our approach to this issue integrated a series of basic and applied studies aimed at gaining a basic understanding of the reproductive physiology of sablefish and developing a method that would eliminate the block or reduce the age of puberty in F1 female sablefish.

Materials and methods

Study I: Assessment of the pituitary gonadotropin-ovary axis in wild maturing and F1 non-maturing female sablefish: To understand the endocrine differences that underlie the reproductive impairment of female sablefish propagated in captivity, we first compared the pituitary gonadotropin-ovary axis of two stocks of sablefish with different reproductive status: wild-caught maturing females and 8 year-old F1 females that had never shown signs of sexual maturation.

Wild female sablefish were caught using sportfishing gear near the mouth of the Quinault River (Washington, USA) in October 2010. The fish were transported to the Manchester Research Station (Port Orchard, Washington, USA) and maintained in tanks supplied with flow-through, sand-filtered and UV-treated seawater. During February 2011, the peak spawning period along the Washington coast (Mason *et al.*, 1983), the wild females showed signs of sexual maturation by ultrasound. F1 sablefish (broodyear 2003) were reared at the Manchester Research Station in net-pens until fall 2010 when

they were transported to the Northwest Fisheries Science Center (NWFSC, Seattle, Washington, USA) where they were reared on recirculated seawater. These females were maintained under similar rearing conditions as the wild-caught fish at the Manchester Research Station. In contrast to the wild females, the F1 females did not exhibit signs of ovarian maturation when assessed by ultrasound.

Wild and F1 females were sampled on 28 February 2011 and 8 March 2011, respectively. Three females from each broodstock group were deeply anesthetized with MS-222 and body weight (BW) and fork length (FL) recorded (wild, 6413.0 ± 712.9 g BW and 81.3 ± 3.1 cm FL; F1, 4160.0 ± 303.3 g BW and 69.8 ± 2.7 cm FL). Plasma was obtained by centrifugation of whole blood and stored at −20 ℃ for sex steroid analyses. The pituitary gland and a small piece of ovary (~80 mg) were also collected from each fish, frozen in liquid nitrogen and stored at −80 ℃ until RNA extraction. Genes of interest included the pituitary gonadotropin subunits (*fshb, lhb, cga*) and ovarian gonadotropin receptors (*fshr* and *lhcgr*). For histology, a middle portion of the ovary was preserved in Bouin's fixative for 48h prior to storage in 70% ethanol and processing as described elsewhere (Campbell *et al.*, 2006).

Study II: Effect of sex steroids on pituitary gonadotropin gene expression *in vivo*: To determine potential effects of sex steroids on pituitary gonadotropins and whether sex steroids will be an important part of hormone therapies to reduce the age of puberty in sablefish, we evaluated the effect of testosterone (T) and estradiol-17beta (E2) on pituitary gonadotropin beta subunit (*fshb* and *lhb*) mRNA levels in prepubertal F1 females *in vivo*.

Twelve 2-year old prepubertal F1 female sablefish (1187.4 ± 49.4 g BW and 469.9 ± 5.3 cm FL) maintained at the NWFSC on 12 ℃ recirculated seawater were distributed into three groups (n=4 sablefish/group) and implanted with cholesterol-based pellets (0.2 x 0.5 mm) containing no hormone (control) or containing T at doses of 0.75 or 3.75 mg. Twenty-eight days after implantation, fish were euthanized and their pituitaries removed and snap frozen for later RNA isolation. In a parallel study, 6 females from the same cohort of fish received 6 intramuscular injections of 2 mg E2/kg dissolved in ethanol: 0.9% NaCl (1:7) or vehicle alone (control), every other day. Two days after the last injection, fish were euthanized and pituitaries removed to determine gonadotropin beta subunit gene expression. Doses of T and E2 were selected from previous studies in other fish species (Guzmán *et al.*, 2008; Holland *et al.*, 2002; Vidal *et al.*, 2004)

Study III: Effect of gonadotropins on ovarian secretion of estradiol *in vitro*: To determine whether immature ovaries of F1 female sablefish are responsive to gonadotropin stimulation, we evaluated the effect of two gonadotropin preparations, recombinant coho salmon Fsh analog (sFsha) and human chorionic gonadotropin (hCG), on E2 production *in vitro*.

For this, a 3-year old prepubertal F1 female sablefish (2910 g BW, 67.6 cm FL) maintained at the NWFSC was euthanized and the ovaries removed. Ovarian fragments (~60 mg each) were cultured in Cortland's solution alone (control) or containing sFsha (provided by Dr. W.R. Moyle, Robert Wood Johnson Medical School, New Jersey, USA) at doses of 50 and 500 ng/ml or hCG (Sigma) at doses of 1, 10 and 100 IU/ml. After 24 h incubation at 11 ℃, the culture medium was collected and stored at -20 ℃ for E2 analysis. Doses of sFsha and hCG were selected based on previous studies in other species (Luckenbach *et al.*, 2011; Sorbera *et al.*, 2001).

RNA isolation, cDNA synthesis and quantitative PCR: Total RNA from sablefish pituitaries and ovarian tissue was isolated with Tri-Reagent (Molecular Research Center) using a TissueLyser II (Qiagen). An aliquot of total RNA was diluted to ~250 ng RNA/μl in nuclease-free water and DNase treated using the DNA Free kit's "rigorous" protocol (Ambion). RNA yields and quality were assessed by NanoDrop (ND-1000 Spectrophotometer) and gel electrophoresis. For reverse transcription, 1 μg of total RNA of each sample was reverse transcribed in a 20-μl reaction with the Superscript II kit (Invitrogen). Quantitative PCRs were conducted as previously described (Luckenbach *et al.*, 2011). Reactions consisted of 1x Power SYBR Green PCR master mix (Applied Biosystems), 150 nM of gene-specific primers (see Guzmán *et al.*, 2013 for primer sequences) and 0.5 ng of pituitary or ovarian cDNA template. Assays were run on an ABI 7700 Sequence

Detector using standard cycling conditions.

Sex steroids: Plasma levels of E2 were measured by radioimmunoassay and levels of T were quantified by enzyme-linked immunosorbent assay using protocols previously validated for sablefish (Guzmán *et al.*, 2013).

Statistical analyses: Statistical analyses were performed using Prism 5 software for Mac OSX (GraphPad Software) with the minimum level of significance set to $P<0.05$. Differences were examined using t-test or one-way ANOVA followed by a Tukey multiple comparisons test. When necessary, data were log or Ln transformed in order to comply with normality and homogeneity of variance, which were tested by Kolmogorov–Smirnov and Bartlett methods, respectively. Data are expressed as mean ± standard error of the mean (S.E.M.).

Results

Study I: Assessment of the pituitary gonadotropin-ovary axis in wild maturing and F1 non-maturing female sablefish

Endocrine parameters indicated a more active reproductive axis in wild versus F1 female sablefish (Fig. 1). At the pituitary level, *lhb* and *cga* mRNA

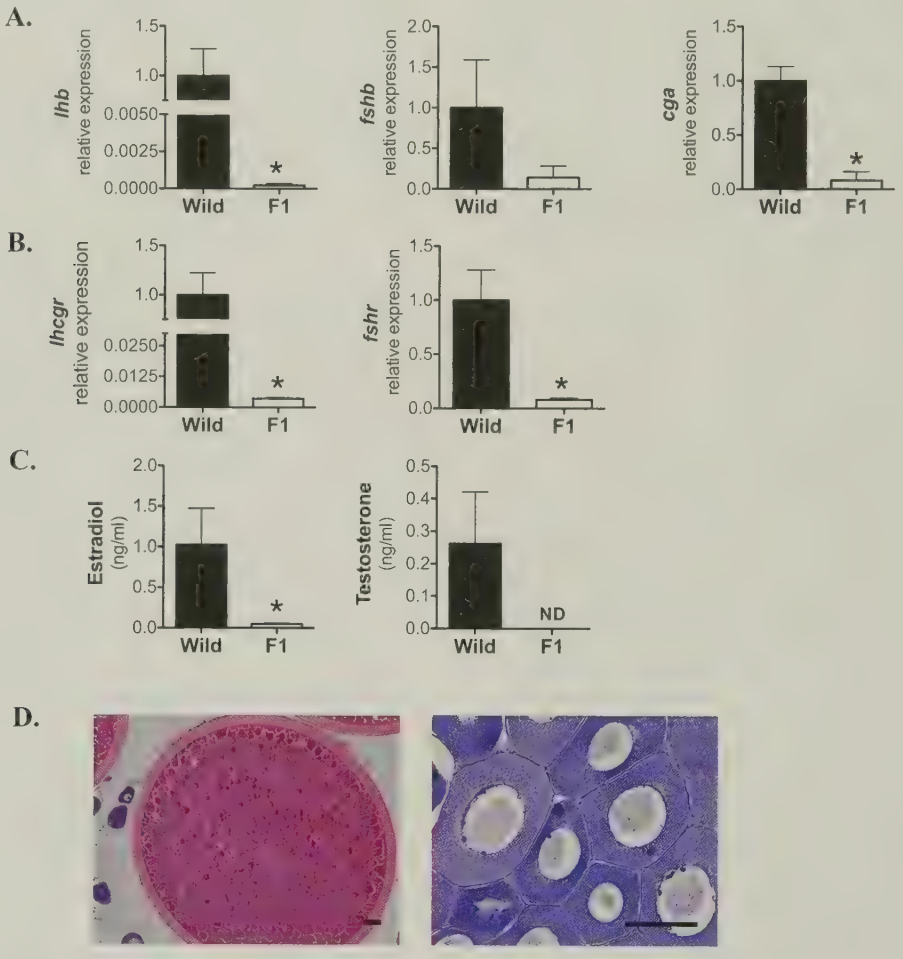


Fig. 1. Relative gene expression of pituitary gonadotropin subunits (A) and ovarian gonadotropin receptors (B), plasma sex steroid levels (C), and representative ovarian histological sections (D) of wild maturing and F1 female sablefish. Gene expression levels were determined by qPCR and normalized to *eef1a*. Levels of E2 were determined by RIA, whereas levels of T were determined by ELISA. Data are expressed as the mean ± SEM ($n=3$). Asterisks indicate significant differences (t-test, $p<0.05$). Maturing follicles with the nucleus migrating to the periphery predominated in the ovaries of wild maturing females, whereas only follicles at the perinucleolus stage were found in ovaries of F1 females (D). Scale bars: 100 μm. ND, non detectable.

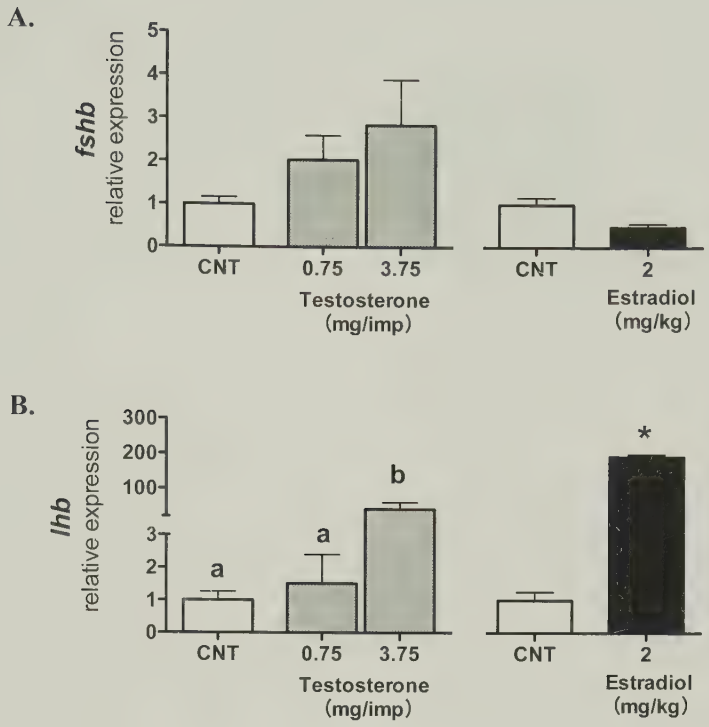


Fig. 2. Relative gene expression of pituitary follicle-stimulating hormone beta subunit (A) and luteinizing hormone beta subunit (B) in prepubertal F1 female sablefish treated with T (left graphs) or E2 (right graphs). For T treatments, females received a single implant containing hormone at doses of 0 (control), 0.75 and 3.75 mg for 28 days. For E2 treatments, females received 6 intramuscular injections of 2 mg hormone/kg dissolved in ethanol: 0.9% NaCl (1:7) or vehicle alone (control, CNT), every other day. Gene expression levels were determined by qPCR and normalized to *eef1a*. For T treatments, bars with different superscript letters are significantly different (ANOVA, $p<0.05$). For the E2 treatment, an asterisk indicates a significant difference relative to control (t-test, $p<0.05$).

levels were significantly higher in wild fish than in F1 fish. Levels of *fshb* were also elevated in wild fish relative to F1 fish (7.1-fold), although differences were not statistically significant due to the high variance across individuals. In the ovary, transcripts for both gonadotropin receptors, *fshr* and *lhcgr*, were significantly higher in wild than in F1 females. Plasma levels of E2 were also significantly higher in wild than in F1 females, whereas only wild females had detectable levels of T in plasma.

The ovary of wild females consisted of post-vitellogenic preovulatory follicles characterized by the nucleus migrating to the periphery and yolk coalescence, as well as another clutch of follicles at the perinucleolus stage. In contrast, ovaries from F1 females were characterized by the sole presence of follicles at the perinucleolus stage.

Study II: Effect of sex steroids on pituitary gonadotropin gene expression *in vivo*

The effect of treatment with T or E2 on *in vivo* pituitary gonadotropin beta subunit (*fshb* and *lhb*) mRNA levels is shown in Fig. 2. Transcripts for *fshb* were not significantly affected by treatment with T or E2. In contrast, transcripts for *lhb* increased significantly in fish treated with the highest dose of T or multiple injections of E2 (40- and 185-fold, respectively, relative to control).

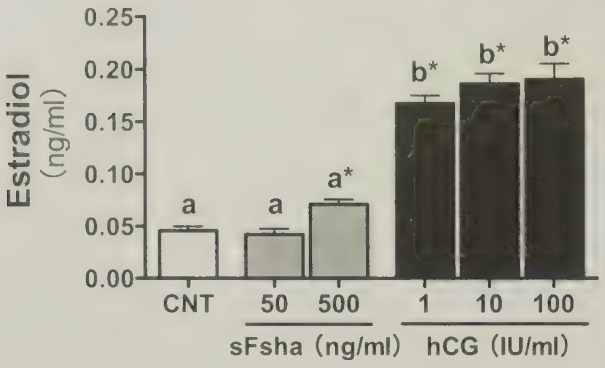


Fig. 3. Effect of sFsha or hCG on E2 production by ovarian fragments obtained from prepubertal F1 female sablefish. Ovarian fragments were cultured in Cortland's solution alone (control, CNT) or containing sFsha at doses of 50 and 500 ng/ml or hCG at doses of 1, 10 and 100 IU/ml, at 11 ℃ for 24h. Levels of E2 were determined by RIA. Bars with different superscript letters are significantly different (ANOVA, $p<0.05$), whereas asterisks indicate significant differences relative to control (t-test, $p<0.05$).

Study III: Effect of gonadotropins on ovarian secretion of estradiol *in vitro*

The effect of sFsha or hCG on ovarian secretion of E2 *in vitro* is shown in Fig. 3. Incubation with sFsha had a subtle effect on the secretion of E2 with only the highest dose of sFsha having a significant effect compared to control. In contrast, treatment with hCG regardless of dose significantly stimulated E2 secretion (>4-fold).

Discussion

Currently, the emerging aquaculture industry for sablefish is mostly dependent on spawning wild-caught individuals. The expansion and optimization of this industry requires a closer understanding of the reproductive physiology of this species and the development of protocols to control the age of puberty in cultured broodstock.

In Study I, we demonstrated that transcript levels of pituitary *lhb* and ovarian *lhcgr* were higher in wild-caught maturing females than in non-maturing F1 females. This is in accordance with the predominant role of Lh in the regulation of gonadal maturation, ovulation and spawning in fishes (Gothilf *et al.*, 1997; Swanson *et al.*, 2003; Yaron *et al.*, 2003). Interestingly, ovarian *fshr* mRNA levels were also higher in maturing females and a similar tendency was observed for the pituitary *fshb* mRNA levels. Little is known about specific roles of Fsh during gametogenesis in fishes, although it is accepted that Fsh is important for early gonadal development and vitellogenesis (Lubzens *et al.*, 2010; Luckenbach *et al.*, 2011). The elevated levels of pituitary *fshb* and ovarian *fshr* that we observed in wild females may indicate that a population of perinucleolar oocytes within the ovary were preparing for the transition to vitellogenic growth, or that Fsh also plays a role in final oocyte maturation. Since the ovary of the wild mature females had a mixed population of oocytes it is not possible to distinguish these two possibilities. Plasma levels of E2 and T were also higher in wild females than F1 females as expected from the observed differences in pituitary gonadotropin subunit and ovarian receptor mRNA levels. This demonstrates classical gonadotropin-mediated sex steroid secretion (Lubzens *et al.*, 2010) by the ovaries of wild females and indicates a lack of gonadotropin signaling in F1 females.

Although F1 females from Study I were 8 years old, they remained arrested in a prepubertal stage. Interestingly, it has been recently demonstrated that some F1 females can initiate vitellogenesis (i.e., onset of puberty) if they are maintained in cold water (~4 ℃) after 5 years of age (B. Campbell, personal communication). It is generally accepted that every fish species has an optimal range of water temperatures for vitellogenin synthesis, and both higher and lower temperatures affect the normal progression of vitellogenesis (Guzmán *et al.*, 2008; Kim and Takemura, 2003). Considering that sablefish is a deep-water species and the onset of vitellogenesis may naturally occur at very low temperatures, it is possible that the temperature at which fish were maintained (fluctuated between 8 and 15 ℃ throughout the year) had a deleterious effect on reproductive axis function, and ultimately on the onset of vitellogenesis in F1 females.

Although some F1 females maintained in cold water can achieve puberty after 5+ years, the development of methods to reduce and synchronize the age of puberty is essential for reliable and cost effective sablefish breeding programs. Hormone

treatments are widely used in aquaculture to improve the reproductive performance of captive fishes, including activation of the reproductive endocrine system and promotion of gonadal development in immature stages (Holland *et al.*, 2002; Zanuy *et al.*, 1999). We conducted Studies II and III to determine the ability of exogenous hormones to stimulate the reproductive axis in prepubertal F1 sablefish.

In Study II we demonstrated that treatment with T or E2 significantly elevated transcripts for pituitary *lhb*, but did not affect levels of *fshb* mRNA. Effects of sex steroids on levels of the pituitary gonadotropin subunit mRNAs have been demonstrated in a number of fishes, although results vary among species. For example, treatment with T elevated pituitary transcripts of *lhb* in European sea bass during the period of "sexual resting," while T and E2 reduced pituitary *fshb* mRNA levels (Mateos *et al.*, 2002). In immature European eel (*Anguilla anguilla*), treatments with E2 but not T elevated transcripts for *lhb*, whereas no effect was observed on *fshb* mRNA levels (Aroua *et al.*, 2007). Lastly, treatments with T and E2 increased pituitary transcript levels for *fshb* and *lhb* in zebrafish (*Danio rerio*) (Lin and Ge, 2009). Our results suggest that pituitaries from prepubertal female sablefish are responsive to exogenous hormones, and that sex steroids may be an important part of a hormone therapy to stimulate the reproductive axis of F1 females. In line with this, treatments with sex steroids were necessary to induce puberty in European sea bass (Zanuy *et al.*, 1999), striped bass (Holland *et al.*, 2002) and European eel (Vidal *et al.*, 2004).

In Study III, we demonstrated that treatment with hCG, an Lh-like gonadotropin, stimulates ovarian secretion of E2 *in vitro*. Treatments with gonadotropin preparations, which act directly on the gonad, have proven effective in the stimulation of gonadal maturation, ovulation and spawning in a number of fish species (Zohar and Mylonas, 2001). Most importantly, in species from the genus *Anguilla*, captive fish often remain arrested in a prepubertal stage in captivity. However, multiple injections with pituitary extracts can trigger ovarian vitellogenesis, maturation and ovulation in females (Ohta and Tanaka, 1997), while multiple injections of hCG can induce testicular development and spermiation in males (Dou *et al.*, 2007). Our data suggest that, as in anguillid eels, immature ovaries of F1 female sablefish are equipped with the ability to synthesize and release sex steroids (critical for vitellogenesis) under appropriate hormone stimulation, and that treatment with gonadotropin preparations may effectively stimulate ovarian function in this species.

In conclusion, wild-maturing females had elevated gonadotropin signaling (pituitary and ovarian gene expression and plasma steroid levels) compared to 8 year-old F1 females, which were holding at the immature, perinucleolus ovarian stage. Anecdotal evidence indicates that F1 females maintained in ~4 ℃ seawater may mature in captivity after 5 years of age. It might be possible that culture conditions that use warmer water (10–15 ℃) suppress the pituitary gonadotropin-ovary axis in sablefish, and ultimately block the onset of puberty. Our results indicate that hormone treatments, such as sex steroids and gonadotropin preparations, can stimulate the reproductive endocrine axis in prepubertal females and therefore should be considered as candidates to reduce the age of puberty in this species.

References

Aizen J., Meiri I., Tzchori I., Levavi-Sivan B., and Rosenfeld H., 2005: Enhancing spawning in the grey mullet (*Mugil cephalus*) by removal of dopaminergic inhibition. *Gen. Comp. Endocrinol.*, **142**, 212–221.

Aroua S., Weltzien F. A., Belle N. L., and Dufour S., 2007: Development of real-time RT-PCR assays for eel gonadotropins and their application to the comparison of in vivo and in vitro effects of sex steroids. *Gen. Comp. Endocrinol.*, **153**, 333–343.

Campbell B., Dickey J., Beckman B., Young G., Pierce A., Fukada H., and Swanson P., 2006: Previtellogenic oocyte growth in salmon: relationships among body growth, plasma insulin-like growth factor-1, estradiol-17beta, follicle-stimulating hormone and expression of ovarian genes for insulin-like growth factors,

steroidogenic-acute regulatory protein and receptors for gonadotropins, growth hormone, and somatolactin. *Biol. Reprod.*, **75**, 34-44.

Dou S. Z., Yamada Y., Okamura A., Tanaka S., Shinoda A., and Tsukamoto K., 2007: Observations on the spawning behavior of artificially matured Japanese eels *Anguilla japonica* in captivity. *Aquaculture*, **266**, 117-129.

Gothilf Y., Meiri I., Elizur A., and Zohar Y., 1997: Preovulatory changes in the levels of three gonadotropin-releasing hormone-encoding messenger ribonucleic acids (mRNAs), gonadotropin beta-subunit mRNAs, plasma gonadotropin, and steroids in the female gilthead seabream, *Sparus aurata*. *Biol. Reprod.*, **57**, 1145-1154.

Guzmán J. M., Luckenbach J. A., and Swanson P., 2013: Molecular characterization and quantification of sablefish (*Anoplopoma fimfria*) gonadotropins and their receptors: Reproductive dysfunction in female captive broodstock. *Gen. Comp. Endocrinol.*, **193**, 37-47.

Guzmán J. M., Norberg B., Ramos J., Mylonas C. C., and Mañanós E. L., 2008: Vitellogenin, steroid plasma levels and spawning performance of cultured female Senegalese sole (*Solea senegalensis*). *Gen. Comp. Endocrinol.*, **156**, 285-297.

Holland M. C. H., Hassin S., and Zohar Y., 2002: The effects of long-term testosterone, gonadotropin-releasing hormone agonist and pimozide treatments on testicular development and luteinizing hormone levels in juvenile and early maturing striped bass, *Morone saxatilis*. *Gen. Comp. Endocrinol.*, **129**, 178-187.

Kim B. H., and Takemura A., 2003: Culture conditions affect induction of vitellogenin synthesis by estradiol-17β in primary cultures of tilapia hepatocytes. *Comp. Biochem. Physiol. Part B: Biochem. Mol. Biol.*, **135**, 231-239.

Levavi-Sivan B., Bogerd J., Mañanós E. L., Gomez A., and Lareyre J. J., 2010: Perspectives on fish gonadotropins and their receptors. *Gen. Comp. Endocrinol.*, **165**, 412-437.

Lin S. W., and Ge W., 2009: Differential regulation of gonadotropins (FSH and LH) and growth hormone (GH) by neuroendocrine, endocrine, and paracrine factors in the zebrafish--An in vitro approach. *Gen. Comp. Endocrinol.*, **160**, 183-193.

Lubzens E., Young G., Bobe J., and Cerdà J., 2010: Oogenesis in teleosts: How fish eggs are formed. *Gen. Comp. Endocrinol.*, **165**, 367-389.

Luckenbach J. A., Dickey J. T., and Swanson P., 2011: Follicle-stimulating hormone regulation of ovarian transcripts for steroidogenesis-related proteins and cell survival, growth and differentiation factors in vitro during early secondary oocyte growth in coho salmon. *Gen. Comp. Endocrinol.*, **171**, 52-63.

Mason J. C., Beamish R. J., and McFarlane G. A., 1983: Sexual maturity, fecundity, spawning, and early life history of sablefish (*Anoplopoma fimbria*) off the Pacific Coast of Canada. *Can. J. Fish. Aquat. Sci.*, **40**, 2126-2134.

Mateos J., Mañanos E., Carrillo M., and Zanuy S., 2002: Regulation of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) gene expression by gonadotropin-releasing hormone (GnRH) and sexual steroids in the Mediterranean Sea bass. *Comp. Biochem. Physiol. Part B: Biochem. Mol. Biol.*, **132**, 75-86.

Mylonas C. C., Fostier A., and Zanuy S., 2010: Broodstock management and hormonal manipulations of fish reproduction. *Gen. Comp. Endocrinol.*, **165**, 516-534.

Ohta H., and Tanaka H., 1997: Relationship between serum levels of human chorionic gonadotropin (hCG) and 11-ketotestosterone after a single injection of hCG and induced maturity in the male Japanese eel, *Anguilla japonica*. *Aquaculture*, **153**, 123-134.

Sorbera L. A., Asturiano J. F., Carrillo M., and Zanuy S., 2001: Effects of polyunsaturated fatty acids and prostaglandins on oocyte maturation in a marine teleost, the European sea bass (*Dicentrarchus labrax*). *Biol. Reprod.*, **64**, 382-389.

Swanson P., Dickey J. T., and Campbell B., 2003: Biochemistry and physiology of fish gonadotropins. *Fish Physiol. Biochem.*, **28**, 53-59.

Taranger G. L., Carrillo M., Schulz R. W., Fontaine P., Zanuy S., Felip A., Weltzien F. A., Dufour

S., Karlsen Ø., Norberg B., Andersson E., and Hansen T., 2010: Control of puberty in farmed fish. *Gen. Comp. Endocrinol.*, **165**, 483-515.

Vidal B., Pasqualini C., Le Belle N., Holland M. C. H., Sbaihi M., Vernier P., Zohar Y., and Dufour S., 2004: Dopamine inhibits luteinizing hormone synthesis and release in the juvenile European eel: A neuroendocrine lock for the onset of puberty. *Biol. Reprod.*, **71**, 1491-1500.

Yaron Z., Gur G., Melamed P., Rosenfeld H., Elizur A., and Levavi-Sivan B., 2003: Regulation of fish gonadotropins. *Int. Rev. Cytol.*, **225**, 131-185.

Zanuy S., Carrillo M., Mateos J., Trudeau V., and Kah O., 1999: Effects of sustained administration of testosterone in pre-pubertal sea bass (*Dicentrarchus labrax* L). *Aquaculture*, **177**, 21-35.

Zohar Y., Muñoz-Cueto J. A., Elizur A., and Kah O., 2010: Neuroendocrinology of reproduction in teleost fish. *Gen. Comp. Endocrinol.*, **165**, 438-455.

Zohar Y., and Mylonas C. C., 2001: Endocrine manipulations of spawning in cultured fish: from hormones to genes. *Aquaculture*, **197**, 99-136.

水研センター研報，第40号，121－128，平成27年
Bull. Fish. Res. Agen. No. 40, 121－128, 2015

Induced Spawning in the Sea Cucumber *Apostichopus japonicus* by Neuropeptide, Cubifrin

Keisuke YAMANO*1, Atushi FUJIWARA*2, Michiyasu YOSHIKUNI*3

Abstract: In hatcheries the induction of spawning in sea cucumbers has been typically carried out by regulation of rearing conditions such as temperature and light intensity. However, this method is relatively ineffective and the rate of spawning is unpredictable. In this study we established an efficient method for inducing spawning in the Japanese sea cucumber *Apostichopus japonicus* by injecting a neuropeptide, cubifrin.

We purified peptides that can induce oocyte maturation from the buccal tissues containing the nerve ring, using liquid chromatography and *in vitro* assay with ovarian fragments in combination. The effective dose of each peptide was evaluated with chemically synthesized peptides. Consequently, the most potent peptide was identified as NGIWY-amide. We also found that synthetic derivatives replaced the third amino acid, isoleucine, with a different basic amino acid could be 10–100 times more potent than the natural hormone.

When injected into the body cavity of sexually matured individuals, NGIWY-amide, or its derivative, induced spawning in both males and females. NGIWY-amide was then named cubifrin after the Japanese word "kubifuri" meaning waving head, which is a reproductive behavior of sea cucumbers. Gamete shedding started about 60 min and 80 min after the injection in males and females, respectively, and was completed almost simultaneously in the two sexes about 2 hours after the administration. The *in vitro* responsiveness of biopsied ovarian fragments was well correlated with the spawning success induced by an injection. Therefore, cubifrin can be used as an excellent detector of maturity as well as an inducer of spawning in *A. japonicus* in a hatchery setting.

Key words: reproduction, hormone, aquaculture, echinoderm

The Japanese common sea cucumber *Apostichopus japonicus* is the most commercially-important holothurian species in Japan. Until recent years the annual catch had been about 5,000–10,000 tons (wet weight) but fishing pressure has been increasing mainly because of a growing demand for export to China. Therefore, the sustainable production of the sea cucumber is crucial. For over 30 years, cultured juveniles of this species have been released into fishing grounds to supplement natural stocks. The current situation concerning sea cucumber fisheries makes this activity more important than ever.

In hatcheries, the induction of spawning in sea cucumbers is typically carried out by regulation of rearing conditions such as temperature, water exchange, and light intensity (Battaglene *et al.*, 2002; Sui, 2004; Wang and Cheng, 2004; Liu *et al.*, 2004; Shao *et al.*, 2006). In Japan, wild-caught *A. japonicus* broodstock are induced to spawn in the dark in tanks of seawater at temperatures that are ~ 5℃ higher than natural seawater (Ito, 1995). However, these methods are relatively ineffective and the rate of spawning is unpredictable. Therefore, more effective method of spawning induction has been

2015年1月30日受理（Received on January 30, 2015）
*1 National Research Institute of Aquaculture, Fisheries Research Agency, Minamiise, Watarai, Mie 516-0193, Japan
E-mail: yamano@fra.affrc.go.jp
*2 National Research Institute of Fisheries Science, Fisheries Research Agency, Fukuura, Kanazawa, Yokohama, Kanagawa 236-8648, Japan
*3 Fishery Research Laboratory, Kyushu University, Fukutsu, Fukuoka 811-3304, Japan

desired.

Starfishes have long been used as model animals for the study of reproductive biology after the finding of spawning-inducing activity in a nerve extract in the middle of the 19th century (Chaet and McConnaughy, 1959). At long last, the nervous substance was purified and identified as a relaxin-like protein (Mita *et al.*, 2009). Since both starfishes and sea cucumbers belong to the Echinodermata, their reproductive mechanism might be driven by similar endogenous factors. Thus, we started the study to discover a spawning-inducing substance in *A. japonicus*, following the strategy employed in the study in starfish.

Here, we report a native neuropeptide potent in inducing oocyte maturation and spawning in *A. japonicus*, and propose a procedure of using the peptide in a hatchery setting.

Materials and Methods

Preparation of neural extract for purification of a spawning-inducing substance: Buccal tissues containing the ring nerve were homogenized in the same volume of ice-chilled 4 N acetic acid containing 0.4 mM β-aminoethyl benzensulfonyl fluoride, 10 μg/ml leupeptin and 4 μM pepstatin A. The homogenates were centrifuged at 15,200 x g for 15 min. at 4℃. The resulting supernatants were then ultracentrifuged at 45,000 x g for 1 hr at 4℃. These supernatants of ultracentrifugation were kept as a crude neural extract in aliquots at -80℃. The crude extract was separated by serial ultrafiltration with AmiconUltra YM30 and YM10 (Millipore). The gonadotropic activity determined by the *in vitro* maturation assay was mainly recovered in the fraction of less than 10K daltons. After YM10 filtration, the filtrate was lyophilized and preserved for a subsequent purification process.

Liquid chromatography: The lyophilized preparation was dissolved in MilliQ water. After centrifugation, the supernatant was applied to a Develosil C8-UG-5 column (20 x 50 mm) and eluted with 80 % acetonitrile containing 0.1 % trifluoro acetic acid (TFA) for desalting. The desalted effluent was lyophilized. The lyophilized extract was fractionated by reversed-phase high performance liquid chromatography (RP-HPLC) using a Develosil RPAQUEOUS-AR-5 column (10 x 250 mm) with a linear gradient of acetonitrile from 10 to 40 % with 0.1 % TFA. Each fraction was lyophilized, then dissolved in 0.5 ml MilliQ water to detect the gonadotropic activity by the *in vitro* maturation assay. The fractions containing the gonadotropic activity were separated by a Develosil RPAQUEOUS-AR-5 column (10 x 250 mm) with a linear gradient of acetonitrile from 10 to 30 % containing 20 mM ammonium acetate (pH 6.0). The fractions were lyophilized and assayed as mentioned above. The active fractions were separated by a Develosil RPAQUEOUS-AR3 column (2 x 250 mm) with a linear gradient of acetonitrile from 15 to 22 % with 0.1 % TFA. The gonadotropic activity was detected in fractions 30 and 33 of the last chromatography step.

Determination of chemical structure of gonadotrophic substances: The fractions 30 and 33 were analyzed for accurate mass values and amino acid sequences by liquid chromatograph-tandem mass spectrometers (Quattro Premier, Waters; nanoFrontier LD, Hitachi High-Technologies) and a protein sequencer (491 cLC, Applied Biosystems).

Preparation of peptides: NGIWY-amide (cubifrin) and its derivatives were chemically synthesized. A stock solution of 1 mM dissolved in MilliQ water was stored at -80℃. Prior to use, it was diluted with filtered seawater to the desired concentrations.

***In vitro* maturation assay**: A portion of ovary was excised through a short incision in the body wall. The excised ovarian tissue was cut into small fragments about 3 mm long. For the purification of a maturation-inducing substance, ovarian fragments were incubated with each fraction for 1.5 hr at 20℃. The gonadotropic activity was determined by germinal vesicle breakdown (GVBD) and also by the incidence of ovulation of oocytes from ovarian fragments.

For the detection of the responsiveness to peptides, ovarian fragments were incubated either with the peptide solution at 100, 10, 1 nM, or 100 pM in filtered seawater or with filtered seawater alone. The test was duplicated for each individual. The incidence of GVBD were scored on a scale of 0-3 in which 3 indicates GVBD in over two-thirds

of oocytes in the fragment; 2, in over one-third of oocytes; 1, in up to one-third of oocytes; 0.5, in a few percentage of oocytes; 0, no response of oocytes. The sum of scores of all treated ovarian fragments from one individual (8 fragments for each individual) was regarded as the oocyte maturation score of that individual. After the assay, oocytes in the control ovarian fragments were mechanically separated by gentle pipetting and the diameters of 20 well-developed oocytes were measured.

Injection of cubifrin and observation of reproductive behavior: Cubifrin solution (10 μM) or filtered seawater was injected into the body cavity of thesea cucumbers; the injection volume was 0.1% of body weight (v/w). Each sea cucumber was then separately placed on the bottom of a 21-l tank, and its behavior was continuously monitored until the completion of spawning, or for 2 hr if no spawning occurred. We recorded the times at which the underside of the water surface was reached, when head waving commenced, and when spawning was started and completed. After the completion of spawning, the number of spawned eggs in 10 ml of tank water was counted to estimate the total number of spawned eggs for each female.

Results and Discussion

Purification of an oocyte maturation-inducing substance: The neural extract was separated by three serial HPLCs (Fig. 1). On the third chromatography, the gonadotropic activity was separated into two independent fractions, Fractions 30 and 33 (arrowheads in Fig. 1c). Both fractions were analyzed by mass spectrometry and protein sequencing. The amino acid sequence of Fraction 30 was NGIWY, and Fraction 33's sequence was QGLFSGV. While the calculated monoisotopic mass values of these sequences were 651.320 and 706.365, analyses by mass spectrometry indicated mass signals of 650.35 and 705.42, respectively. Further *de novo* sequencing analyses of these mass signals suggested that these had the same sequences as those given through protein sequencing except for anamidation at each C-terminal end of the peptides. With these analyses we determined that the sequences of the components in Fractions

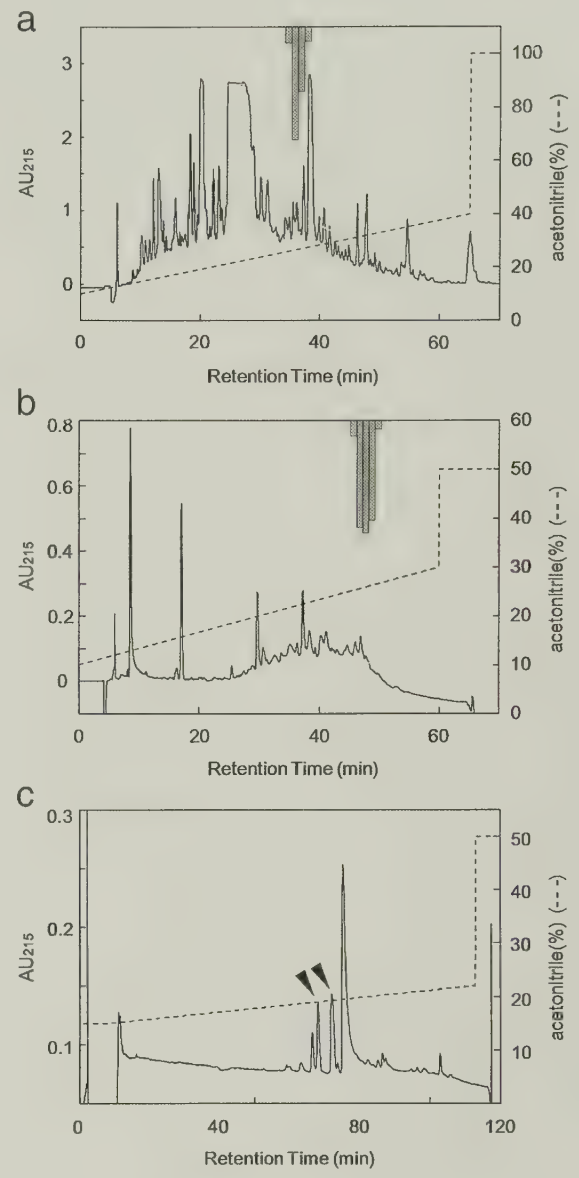


Fig. 1. Separation of the gonadotropic activity by RP-HPLC. Dotted lines in the chromatograms indicate changes of programmed concentrations of acetonitrile in the eluents. Hormonal activity in each fraction is shown with the help of bars hanging from the top side of the chromatograms (a, b). Arrowheads in (c) indicate fractions 30 and 33 containing the bioactivity. Reproduced from Kato *et al.* (2009) with permission.

30 and 33 were NGIWY-amide and QGLFSGV-amide, respectively. Unexpectedly, both of these peptides completely differ from a relaxin, which is an oocyte maturation-inducing substance in starfish. NGIWY-amide has been reported as a peptide associated with contractions of muscle, intestine and tentacles in *A. japonicus* (Inoue *et al.*, 1999),

whereasQGLFSGV-amide has not been reported ever.

***In vitro* maturation-inducing activity of peptides:** Two peptides, NGIWY-amide and QGLFSGV-amide, were chemically synthesized and GVBD-inducing activity was estimated with *in vitro* maturation assay. NGIWY-amide was extremely potent even at 1 nM or less (Fig. 2). QGLFSGV-amide was less potent in inducing GVBD, with effective concentrations being 1 μM or more. Thus, NGIWY-amide is strongly suspected to be involved in the regulation of oocyte maturation. In contrast, QGLFSGV-amide does not appear to be the primary endocrine regulator of oocyte maturation.

The activities of the two derivatives, NGLWY-amide and NGIWY-COOH, were compared with that of the natural NGIWY-amide (Fig. 3). NGLWY-amide was effective at 10 pM or less, a hundred times more potent than the natural peptide, NGIWY-amide. However, NGIWY-COOH barely induced GVBD at 100 nM. Accordingly, C-terminal amidation of the peptide is indispensable for the bioactivity.

Effect of NGIWY-amide and NGLWY-amide in inducing spawning: NGIWY-amide and NGLWY-amide were injected into sexually matured males and females to examine the effect on inducing spawning behavior. Injections of NGIWY-amide induced spawning in males and females at 10 nM. NGLWY-amide more effectively induced spawning in males at 1 nM and in females at 100 pM.NGLWY-amide was therefore at least ten times more potent than NGIWY-amide, as in the case of *in vitro* experiments. Sea cucumbers injected with NGIWY-amide or NGLWY-amide exhibited reproductive behaviors that were independent of sex and typically included: (1) climbing up the side wall of the tank to the underside of the water surface; (2) throwing back and swaying of the head; (3) shedding gametes from the gonopore in the head region. Gamete release occurred 45–60 min after

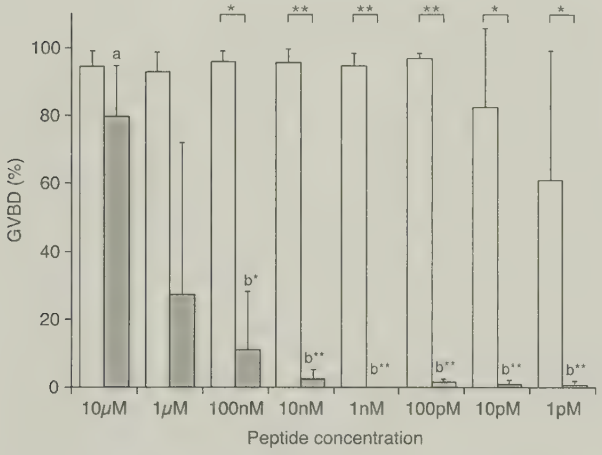


Fig. 2. Concentration dependence of NGIWY-amide and QGLFSGV-amide to induce GVBD in ovarian fragments. Ovarian fragments were incubated with NGIWY-amide (white bars) and QGLFSGV-amide (gray bars) at various concentrations. Bars represent percentages of GVBD (means ± SD of duplicate determinations of four separate experiments). Bars with different labels differ significantly ($P < 0.05^{*}$, $P < 0.01^{**}$). The grouping symbol at the top side of the graph indicates a significant difference between NGIWY-amide and QGLFSGV-amide at each concentration. Significant differences were determined using Wilcoxon's rank sum test. Reproduced from Kato *et al.* (2009) with permission.

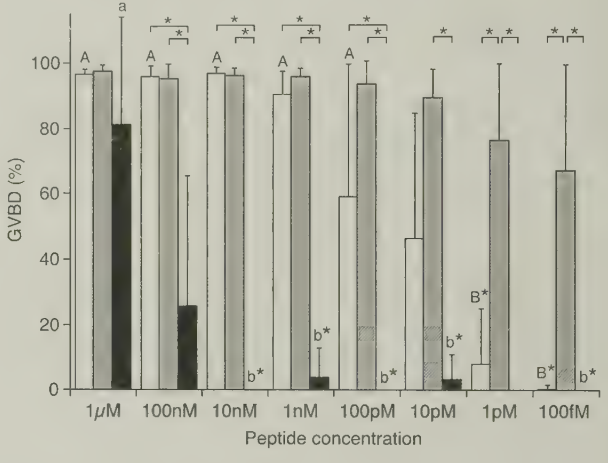


Fig. 3. Concentration dependence of NGIWY-amide and its derivatives to induce GVBD in ovarian fragments. Ovarian fragments were incubated with synthetic analogues at various concentrations. NGIWY-amide (white bars), NGLWY-amide (gray bars) and NGIWY-COOH (black bars) were examined. Bars represent percentages of GVBD (means ± SD of triplicate determinations of six different experiments), and bars with different labels differ significantly in each peptide ($P < 0.05^{*}$, $P < 0.01^{**}$). The grouping symbol at top side of the graph indicates a significant difference among two peptides at each concentration. Significant differences between means were determined using Steel-Dwass's pair-wise comparisons. Reproduced from Kato *et al.* (2009) with permission.

the peptide injections in males and 70–90 min in females, and was completed almost simultaneously in the two sexes about 2 hr after the administration (Fig. 4). This series of actions from climbing to spawning observed in the experiment seemed to be a typical behavior of spawning by sea cucumbers (McEuen, 1988; Battaglene, 2002). NGIWY-amide and NGLWY-amide were named cubifrin and cubifrin-L, respectively, after the Japanese word "kubifuri" meaning waving head, which is a reproductive behavior of sea cucumbers.

Association between oocyte size and responsiveness to cubifrin: The competence of ovarian fragments to undergo *in vitro* oocyte maturation and the induction of spawning by cubifrin-L stimulation were examined in relation to oocyte size. All ovarian fragments with oocytes >155μm in diameter (animal nos. 17–25 in Fig. 5) responded to cubifrin-L *in vitro*, as indicated by their oocyte maturation score, which was a measure of their ability for ovulation and GVBD. In contrast, all fragments but one (animal no. 11 in Fig. 5) with oocytes <155 μm failed to respond to the treatment. The injection of cubifrin-L into the body cavity induced spawning in all females with ovaries that responded to cubifrin-L *in vitro*. Females with unresponsive ovaries *in vitro* did not spawn following the injection, with the exception of two females (nos. 12 and 13). The number of spawned eggs per 100 g body weight varied from 765 to 1,826,000, and there was a significant correlation between this value and the oocyte maturation score (r = 0.84). The number of eggs spawned by the two non-responsive females in *in vitro* experiment was especially low (765 eggs per 100 g body weight in female no. 12 and 23,700 in female no. 13).

These results indicate that potential spawners in broodstock can be selected by *in vitro* examination of the cubifrin or cubifrin-L responsiveness of ovarian fragments or by measuring oocyte size. Furthermore, the number of spawned eggs can be roughly estimated by *in vitro* examination.

Procedure of spawning induction in a hatchery: In conclusion, spawning induction by cubifrin is an

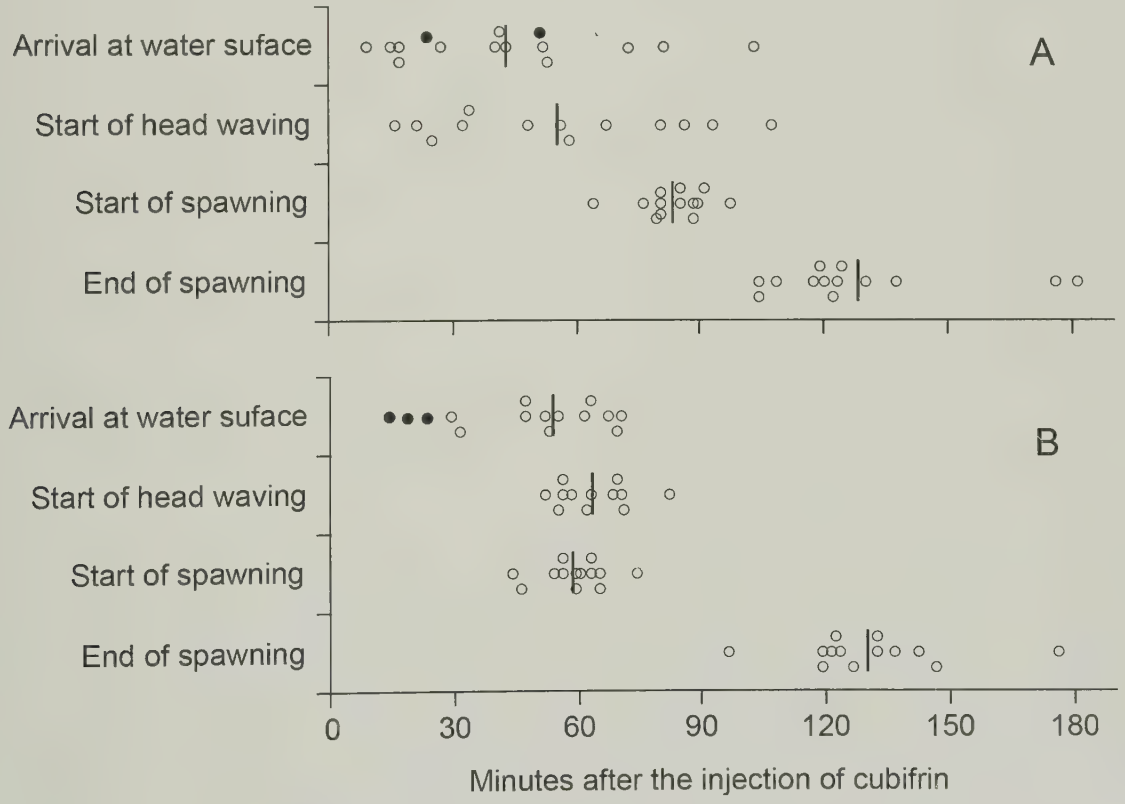


Fig. 4. Progress of spawning behavior in females (A) and males (B). Open and closed circles indicate the data point of each NGLWY-amide-injected male and female (n=14 each) and control sea cucumber (n=4 each). Vertical bars show the averageofthe NGLWY-amide-injected group.

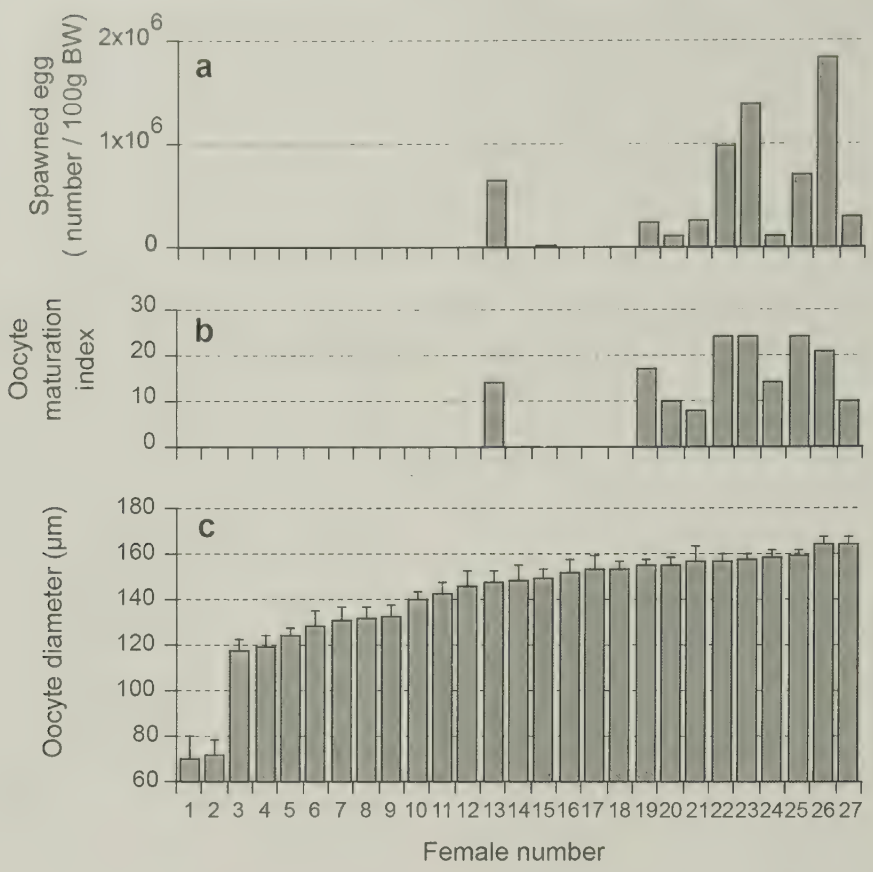


Fig. 5. Association between oocyte size and responsiveness to cubifrin. Responsiveness to NGLWY-amide was determined by spawning success, as indicated by the number of spawned eggs per 100 g body weight (a) and by *in vitro* maturation assay, evaluated from the oocyte maturation score (b). See the Materials and methods for a description of the oocyte maturation score. Oocyte sizes (mean diameter ±SD, n = 20) of each female are shown in (c).

effective and simple method for practical application during the artificial culture of *A. japonicus*. We propose the procedure of spawning induction as follows:

(1)preparation of sea cucumbers during the spawning season; (2)sex determination and gross evaluation of maturity from the appearance of biopsied gonad; (3) evaluation of definite maturity with biopsied ovary by *in vitro* test; (4) Rearing mature individuals below 15℃ to suppress unanticipated natural spawning until spawning induction; (5)spawning induction by the injection of 10μM cubifrin solution (0.1% of body weight (v/w)); (6) standard fertilization and cultivation of fertilized eggs.

Acknowledgements

This work was supported by grants from the Program for Promotion of Basic Research Activities for Innovative Biosciences (PROBRAIN).

References

Battaglene S., Seymour J., Romafafia C., and Lane I., 2002: Spawning induction of three tropical sea cucumbers, Holothuria scabra, *H. fuscogilva* and *Actinopyga mauritiana*. *Aquaculture*, **207**, 29-47.

Chaet A. B., and McConnaughy R. A., 1959: Physiologic activity of nerve extracts. *Biol. Bull.*, **117**, 407-408.

Inoue M., Birenheide R., Koizumi O., Kobayakawa Y., Muneoka Y., and Motokawa T., 1999:

Localization of the neuropeptide NGIWY-amide in the holothurians nervous system and its effects on muscular contraction. *Proc. R. Soc. Lond. B*, **266**, 993-1000.

Ito S., 1995: Study on the technological development of the mass production for sea cucumber juvenile, *Stichopus japonicus* (in Japanese). Saga Prefectural Sea Farming Center.

Kato S., Tsurumaru S., Taga M., Yamane T., Shibata Y., Ohno K., Fujiwara A., Yamano K., and Yoshikuni M., 2009: Neuronal peptides induce oocyte maturation and gamete spawning of sea cucumber, *Apostichopus japonicus*. *Dev. Biol.*, **326**, 169-176.

Liu X., Zhu G., Zhao Q., Wang L., and Gu B., 2004: Studies on hatchery techniques of the sea cucumber, *Apostichopus japonicus*. In: Lovatelli A *et al* (eds) Advance in sea cucumber aquaculture and management, vol **463**. FAO fisheries technical paper, Rome, pp 287-295.

McEuen F., 1988: Spawning behaviors of northeast pacific sea cucumbers (Holothuridae: Echinodermata). *Mar. Biol.*, **98**, 565-585.

Mita M., Yoshikuni M., Ohno K., Shibata Y., Paul Prasanth B., Pitchayawasin S., Isobe M., and Nagahama Y., 2009: A relaxin-like peptide purified from radial nerves induces oocyte maturation and ovulation in the starfish, *Asterina pectinifera*. *PNAS*, **106**, 9507-9512.

Shao M., Zhang Z., Yu L., Hu J., and Kang K., 2006: Cryopreservation of sea cucumber *Apostichopus japonicus* (Selenka) sperm. *Aquacult. Res.*, **37**, 1450-1457.

Sui X., 2004: The progress and prospects of studies on artificial propagation and culture of the sea cucumber, *Apostichopus japonicus*. In: Lovatelli A *et al* (eds) Advance in sea cucumber aquaculture and management, vol **463**. FAO fisheries technical paper, Rome, pp 273-276.

Wang R., and Cheng Y., 2004: Breeding and culture of the sea cucumber, *Apostichopus japonicus*, Liao. In: Lovatelli A *et al.* (eds) Advances in sea cucumber aquaculture and management. FAO fisheries technical paper **463**. FAO, Rome, pp 277-286.

Annotated Bibliography

Kato S., Tsurumaru S., Taga M., Yamane T., Shibata Y., Ohno K., Fujiwara A., Yamano K., and Yoshikuni M., 2009: Neuronal peptides induce oocyte maturation and gamete spawning of sea cucumber, *Apostichopus japonicus*. *Dev. Biol.*, **326**, 169-176.

Extracts prepared from tissues containing buccal ring nerve or longitudinal radial nerve of sea cucumber induce oocyte maturation and ovulation from ovarian tissues. We purified two small peptides, a pentapeptide and a heptapeptide, from the buccal tissues of Japanese common sea cucumber, *Apostichopus japonicus*. Both peptides induced oocyte maturation and gamete spawning. The pentapeptide was identified as NGIWY-amide. This peptide induced *in vitro* germinal vesicle breakdown and ovulation of fully- grown oocytes at less than 1 pM and *in vivo* spawning at 10 nM. A synthetic derivative of the pentapeptide, NGLWY-amide, was 10-100 times more potent compared to the natural NGIWY-amide. The heptapeptide was less potent, inducing ovulation at 1 μM. NGIWY-amide and NGLWY-amide induced a characteristic spawning behavior when injected into sexually matured individuals. Mature eggs artificially spawned were fertilized, and developed normally and metamorphosed into young sea cucumbers. The details of the production and the mechanism of action of NGIWY-amide are still unclear, but the high biopotency of the peptide will aid understanding of the neuronal and hormonal control of reproduction of sea cucumber.

Fujiwara A., Yamano K., Ohno K., and Yoshikuni M., 2010: Spawning induced by cubifrin in the Japanese common sea cucumber *Apostichopus japonicus*. *Fish. Sci.*, **76**, 795-801.

The neuropeptide cubifrin-I and its derivative cubifrin-L have recently been demonstrated as potent substances that induce oocyte maturation *in vitro* and spawn in the Japanese common sea cucumber *Apostichopus japonicus*. Here, the reproductive behavior provoked by injection of cubifrin-L into the body cavity of *A. japonicus* was examined with a view to the practical application

of the peptide for induction of spawning in the hatchery. Ovarian fragments with oocytes larger than 155 μm in diameter responded to cubifrin-L *in vitro*. The *in vitro* responsiveness of ovarian fragments was well correlated with the spawning success induced *in vivo* by a cubifrin-L injection. Mature sea cucumbers injected with cubifrin-L displayed sequential reproductive behaviors, which comprised climbing the side wall of the tank toward the water surface, waving of the head, and shedding of gametes. Gamete shedding started about 60 min and 80 min after the injection in males and females, respectively, and was completed almost simultaneously in the two sexes about 2 hours after the administration. Repeated injections of cubifrin-L at intervals of about 10 days successfully induced multiple spawnings in males and females. This study demonstrated that cubifrin-L is an effective inducer of spawning in Japanese sea cucumber cultivation.

水研センター研報，第40号，129－134，平成27年
Bull. Fish. Res. Agen. No. 40, 129－134, 2015

Mass Production of Artificial Seed of the Japanese Common Sea Cucumber (*Apostichopus japonicus*) in Hokkaido, Japan

Yuichi SAKAI*1

Abstract: Seed production and release of *Apostichopus japonicus* is a key approach to increase and maintain their natural stocks in Japan. Nevertheless, in many trials conducted mainly in Honshu and Kyushu by the end of the 1990's, the amount of seed production fluctuated annually, and the survival rate of released artificial juveniles was uncertain because of the difficulty of distinguishing them from wild ones. Due to these issues, some hatcheries discontinued *A. japonicus* enhancement projects. However, due to increase in Chinese demand, the price of *A. japonicus* dramatically increased after 2003 in Hokkaido. Facing the decline in the price of sea urchin and abalone, which are major high-value catches in the coastal areas, fishermen were very interested in *A. japonicus* stock enhancement by releasing artificial seeds. Accordingly, sea urchin and abalone hatcheries in Hokkaido began to produce *A. japonicus* seed after 2006. This paper will introduce the recent mass production techniques developed in Hokkaido.

Key word: *Apostichopus japonicus*, artificial seed production, larval rearing, post-larval rearing, control of predation

Japanese common sea cucumber *Apostichopus japonicus* is found in shallow coastal bottoms of Japan, Kuril Islands, Sakhalin, northeastern China and Korean peninsula. Among these countries, Japanese and Korean people prefer to taste this mainly in raw or picked as a seafood, but Chinese people prefer dried sea cucumber as a tonic rather than a seafood. Due to the economic development of China, demand for this species has increased. Unit price in Hokkaido peaked at 43.2 USD/kg in 2010. Facing the decline in the price of sea urchin and abalone, fishermen were very interested in stock enhancement by releasing artificial seeds.

After Imai *et al.* (1950) made the first trial of artificial seed production in Japan, many trials were conducted in Honshu and Kyushu. Small juveniles (<2mm) were mainly produced by 1990, and the productions peaked at 26.6 million year^{-1}. Thereafter, due to increase of production of larger juveniles, the total number of juveniles decreased to around 2–3 million year^{-1} (Fig. 1).

Mass mortality during post-larval rearing resulted in fluctuations in the number and size of seed produced. Furthermore, the survival rate of released artificial seeds had not been properly

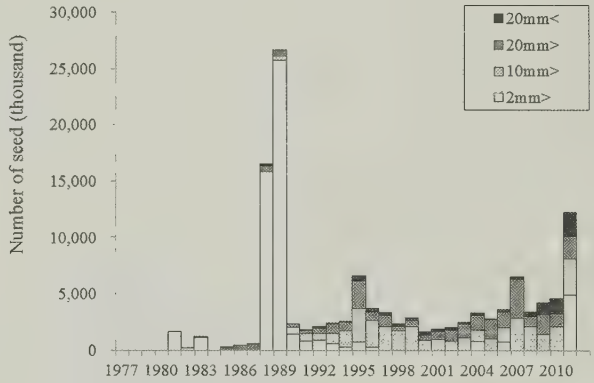


Fig. 1. Number of seed produced in Japan. Drawn from the statistical data published by Fisheries Agency, Fisheries Research Agency and National Association for Promotion of Productive Seas.

2015年1月30日受理 (Received on January 30, 2015)
*1 Fisheries Research Department Mariculture Fisheries Research Institute, Hokkaido Research Organization, 1-156-3, Funami-cho, Muroran, Hokkaido, 051-0013, Japan
E-mail: sakai-yuichi@hro.or.jp

evaluated, because of difficulty in distinguishing cultured from wild individuals. Therefore, some hatcheries in Honshu and Kyushu discontinued this stock enhancement project. However the demand for artifical seed production by fishermen, began to increase after 2006 in Hokkaido and the production of artificial seed increased again and reached to 12.3 million in 2011. From this experience and background, the improvement of artificial seed production is necessary for stock enhancement to be successful.

Seed production of *A. japonicus* is divided into 5 processes: broodstock collection, fertilization, larval rearing, settlement and post-larval rearing. I review the recent mass production techniques mainly developed in Hokkaido for each process.

Brood stock collection

Genetic impacts due to release of artificially produced organisms are considered in broodstock collection. Polymorphic microsatellite allele frequency of artificial seed tends to decrease to compare with wild ones due to small number of their parents (Sakai and Kanno, 2013). So to keep enough broodstock from wild stocks is necessary especially in the case of survival rate of released seed is high enough to contribute its reproduction on the released bottoms. Furthermore, to maintain genetic diversity, broodstock should not be used repeatedly.

Maturation of this species begins after they grow to ca. 50g in body weight, so larger broodstock are necessary. They are normally collected in the spawning season, late June to August in Hokkaido, to avoid excess labor and cost to rear them.

Fertilization

Gametes are obtained from fully matured broodstock by two methods: thermal stimulation or injection of gonadotrophic hormone (CUBIFRIN) through the body wall (Kato *et al.*, 2009; Fujiwara *et al.*, 2010). To induce to release gametes by thermal stimulation, broodstock are placed in 15 L volume containers individually and the seawater temperature is raised by 5–7℃ above ambient temperature. Mature broodstock begin to release their gametes into the container ca. 0.5–1.5 hours after the stimulation. Alternatively, broodstock injected with gonadotrophic hormones (CUBIFRIN) will also begin to release their gametes.

For insemination, 10–20 ml of sperm is added to a liter of seawater containing 0.1 – 0.2 million eggs. To prevent polyspermy, sperm concentration should not exceed 1,000,000 sperm ml^{-1} (Sakai and Konda, 2006). After insemination, eggs are transferred onto a 45μm mesh sieve immersed in a shallow tray and gently rinsed with filtered seawater to wash the excess sperm through the sieve.

Larval rearing

Hatched blastulae swim to the surface and are transferred to larval rearing tanks at a density of 1–2 individuals ml^{-1}. About 2 days after hatch, the larvae grow to the auricularia larvae and begin to eat *Chaetoceros gracilis*, unicellular phytoplankton. Daily feeding rate is near to 10 000 cells $individual^{-1}$. The fully developed auricularia larvae grow to ca. 1 mm body length in 5 to 10 days. After this, they begin to shrink to 0.4–0.5mm doliolaria larvae in 2 days. Then about 1 day after, doriolaria larvae grow to pentactula larvae which are ready to metamorphose to young juveniles and begin to settle onto the tank bottom or collectors.

The growth rate of larvae is regionally different in Hokkaido, fast in southwestern Sea of Japan areas and slow in Pacific areas. It takes only 9 days in southwestern Hokkaido and 14 days after hatch in the Pacific area.

Larval rearing tanks are static through the rearing period to minimize the loss of food and satisfy their food requirement. However, to avoid precipitation of larvae, food and feces on the bottom, aeration is very important; the optimum rate is 1.5–2.0 L min^{-1}. The water temperature should be kept at 18–20℃ .

Settlement

Doriolaria and pentacutula larvae are transferred to post-larval rearing tanks. They settle on corrugated polyvinyl chloride (PVC) plates or balled 1mm mesh polyethylene screens of which surface are covered with natural attached diatoms or algal

powder which are used as the juvenile diet. To avoid the loss of these larvae, water exchange is initiated 10 days after the larvae are stocked, by which time most larvae complete settlement.

Post-larval rearing

Settled juveniles are reared up to 5 to 10 mm body length on corrugated PVC plates and/or polyethylene screens without temperature control. Daily water exchange rate varies from 1-7 times day^{-1} with aeration at the center of the tank to stir the rearing water well.

Various diets during this post-larval rearing have been examined (Yanagibashi *et al.*, 1984; Yanagibashi and Kawasaki, 1985; Ueki and Ikeda, 1989; Konda and Sakai; 2005). The naturally occurring attached diatoms and LIVIC-BW, a commercially sold diet made from a mixture of dried algal powder of *Undaria pinnatifida* and *Ascophyllum nodosum* are used as inducers for larval settlement and as food for post-larval stages. Phytoplankton *C. gracilis* is also a good initial food for juveniles for the first month (Sakai and Konda, 2008). Proper feeding of LIVIC-BW has been examined according to juvenile size (Ikeda *et al.*, 1992; Sakai *et al.*, 2009). Juveniles fed LIVIC-BW grow to 6.0 mm in about 3 months (Sakai *et al.*, 2009). Growth rate affects rearing density (Hatanaka, 1996; Sakai *et al.*, 2009) and food densities (Ito and Kawahara, 1993; Sakai *et al.*, 2009).

During the Great East Japan Earthquake, the factory of LIVIC-BW was destroyed. Argin-Gold (Andes-Trading Co., Ltd.) made from dried powder of *Ascophyllum nodosum* began to be used. This diet compensates the same growth of juveniles at least 3 month (Sakai, unpublished data). Further experiments are necessary to find the proper diet to increase the growth rate of artificial seeds.

In this stage, the predation of settled juveniles by *Tigriopus japonicus*, which is commonly found in the coastal area of western north-Pacific Ocean, was a major problem to produce the artificial seeds in Japan (Kobayashi and Ishida, 1984). This species grow to only ca. 1 mm in its body length as adults and produce more than 100 eggs at each spawn (Fig. 2). Even just hatched out nauplius larvae, 50 μ m in its body length, begin to eat *A. japonicus* juveniles.

The predatory impacts by *T. japonicus* are serious during post-larval rearing. The predation begins after the density of this animals increase to ca. 10-20 individuals $10ml^{-1}$ (Sakai and Konda, 2008;

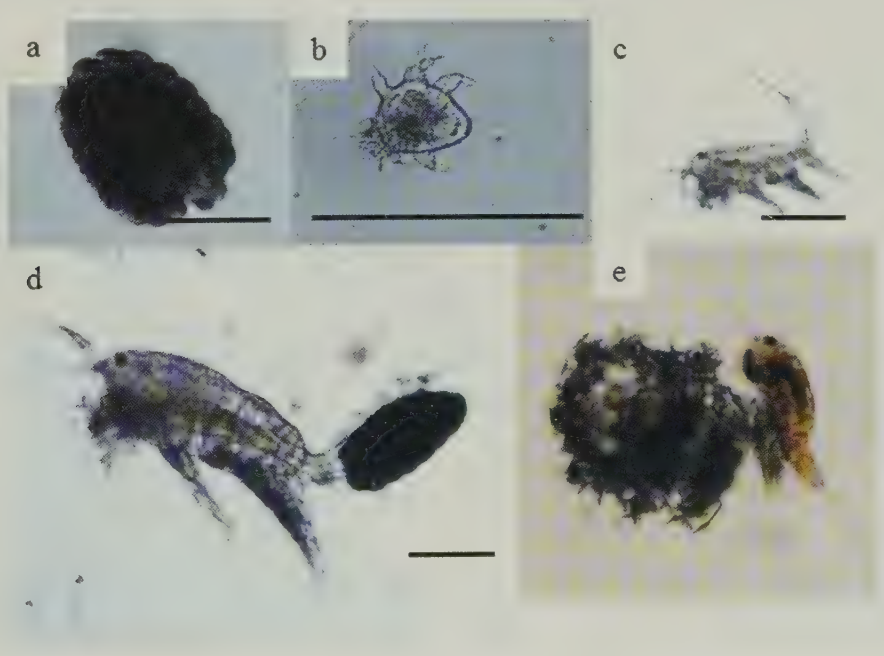


Fig. 2. Development of *Tigriopus japonicas*. a: eggs in the sac, b: higher magnification of fertilized nauplius larva, c: copepodid stage larva, d: adult female holding egg sac, e: pradation of *A. japonicus* juvenile. bar : 250μm.

Kobayashi and Yamaguchi, 2011; Noguchi and Noda, 2011). To prevent predation of artificial juveniles, it is necessary to decrease the density of *T. japonicus*. Trichlorphon, organo-phosphorous compound at a concentration of 1–2 ppm has been used to control this animal (Kobayashi and Ishida, 1984). However, after 2006, this medicine was prohibited by the Pharmaceutical Affairs Law in Japan.

After this, I developed 3 methods to prevent the predation: to immerse the plates into salt enriched seawater (ca.50‰), pumping the rearing water containing this predatory animal and filtered out with a 45μm mesh net to decrease copepod density in the water, and using a balled 1 mm mesh as the settlement material which allows the juvenile sea cucumbers to escape from the copepods (Fig. 3).

Immersing salt enriched water: Hypersaline seawater induces paralysis of the copepods and removes them from the plates, thereby decreasing predation (Sakai and Konda, 2008). Daily observation to confirm the increase of *T. japonicus* and to determine the timing of this treatment is necessary. Furthermore, a new tank free of copepods to set the collectors in is also necessary.

Pumping up to filter copepods: To reduce labor, I set an underwater pump on the bottom of the rearing tank to pump the copepod rich rearing water. The pumped water discharged into a 45 μm mesh net set at the surface of the rearing tank. *T. japonicus* were filtered out by this method and the density of copepods in the rearing tank decreased automatically.

Balled screen collector: The balled polyethylene screen is also available as the collector and rearing device to prevent predation. Sea cucumber juveniles can escape into the center of the balled screen if the

Fig. 3. Three main methods to control copepod density in the post-larval rearing tanks. a: Immersing the plates into salt enriched sea water, b: Pumping up and filter out the copepods, c: Balled screen collector. N: 45μm mesh net, P: under water pump.

density of copepods increases and allows them to escape from predation.

Release of artificial seeds

Juveniles are released onto the reefs by divers. Mitsunaga and Matsumura (2004) reported that large juveniles (26mm) survive better than smaller ones (14mm). However, the survival rate one year after releasing was only 3.8% and 0.1%, respectively.

Sakai and Kanno (2013) estimated the residual rate in an experimental area using msDNA marker. The residual rate of 8mm juveniles was only 3.9% four years after releasing, but the released juveniles had also observed in the catches from the fishing area neighbor to this experimental area.

Future perspective

To succeed in increasing the stock of sea cucumbers, further improvement in seed production, especially improving feeding to promote growth during post-larval rearing, is necessary. In addition, evaluating the effectiveness of artificial seed release strategy is indispensable.

The cheapest juvenile for release onto the fishery ground is the newly settled size, 0.4 mm in body length. Fishermen can produce these small juveniles themselves even in the corner of the port without many devices, technicians or long term labor. We can now distinguish even such a small seed from wild ones by msDNA markers. We will now determine if this small seed contributes to increase the natural stocks. The size dependence of the recapture rate should be evaluated as soon as possible for this project to succeed.

References

Hatanaka H., 1996: Density effects on growth of artificially propagated sea cucumber *Stichopus japonicus* juveniles. *Suisan Zoushoku*, **44(2)**, 141–146 (in Japanese with English abstract).

Ikeda Z., Ueki N., and Kusaka K., 1992: On the proper volume of the diet LIVIC-BW given to the juvenile sea cucumber *Stichopus japonicus*. *Bull. Fish. Exp. Station Okayama Pref.*, **7**, 53–55 (in Japanese).

Imai T., Inaba D., Sato R., and Hatanaka S., 1950: Mushoku Benmouchu niyoru Namako (*Stichopus japonicus*) no Jinkoushiiku (Artificial seed production of sea cucumber, Stichopus japonicus, using non-pigmented flagellates). *Bull. Inst. Agricultural Res. Tohoku Univ.*, **2(2)**, 269–277 (in Japanese).

Ito S., and Kawahara I., 1993: Manamako no futyakukeisouban siiku niyoru tairyouseisan (Mass production of sea cucumber juveniles using benthic diatoms). *Bull. Saga Pref. Sea Farming Center*, **2**, 1–11 (in Japanese).

Kato S., Tsurumaru S., Taga M., Yamane T., Shibata Y., Ohno K., Fujiwara A., Yamano K., Yoshikuni M., 2009: Neuronal peptides induce oocyte maturation and gamete spawning of sea cucumber, *Apostichopus japonicus*. Dev. Biol., **326**, 169–176.

Kobayashi M., and Ishida M., 1984: Chinamako no genmouyouin ni kansuru nisan no jikken (Some experiments on the cause of death of sea cucumber juveniles). *Saibai Giken*, **13(1)**, 41–48 (in Japanese).

Kobayashi T., and Yamaguchi H., 2011: Effects of *Tigriopus japonicus* for seedling production of Japanese sea cucumber *Apostichopus japonicus*. *Bull. Iwate Pref. Fish. Tech. Center*, **7**, 19–24 (In Japanese with English abstract).

Konda Y., and Sakai Y., 2005: An Experimental study to observe the gut contents of juvenile sea cucumber, *Apostichopus japonicus*. *Sci. Rep. Hokkaido Fisheries Experimental Station*, **68**, 71–74 (in Japanese with English abstract).

Mitsunaga N., and Matsumura Y., 2004: Growth and survival of hatchery-produced juveniles of sea cucumber *Apostichopus japonicus* in different release size. *Bull. Nagasaki Pref. Institule Fisheries*, **30**, 7–12 (in Japanese with English abstract).

Noguchi K., and Noda S., 2011: Influence of Copepods which occur during seed production of the sea cucumber, *Apostichopus japonicus*. *J. Fisheries Technology*, **3(2)**, 131–135 (In Japanese with English abstract).

Sakai Y., and Kanno M., 2013: DNA kaiseki niyoru manamako no houryukouka suiteigijyutsu no

kaihatu to keigunkouzou no kaimei (Estimation of the effectiveness of artificial seed releasing and population structure of *Apostichopus japonicus* using DNA analysis). *Annual report of Mariculture Fisheries Research Institute* 6-18 (in Japanese).

Sakai Y., and Konda Y., 2006: Kairui syubyouseisan gijyutsukaihatsu-Manamako- (Development of mass production technique of shellfish - *Apostichopus japonicus*-) *Annual report of Hokkaido institute of Mariculture* 16-38. (in Japanese)

Sakai Y., and Konda Y., 2008: Manamako Jinkousyubyou no Syokugai Boujyogijyutsu nituite (New mass production technique to prevent the predation of artificial juveniles from copepods) *Hokusuishi Dayori* **76**, 8-14 (in Japanese).

Sakai Y., and Konda Y., 2008: Manamako jinkousyubyou no rikujyou ikuseigijyutsu kakuritusiken (Experimental study of rearing techniques of *Apostichopus japonicus* in land base hatcheries) *Annual report of Hokkaido Mariculture Fisheries Experiment Station*, 81-93 (in Japanese).

Sakai Y., Konda Y., Nakajima K., and Gohda H., 2009: Manamako Jinkousyubyou no Rikujyoikusei Manyuaru (Manual for the seed production of *Apostichopus japonicus* in landbased hatcheries). *Hokkaido Mariculture Fisheries Experiment Station, Muroran, Hokkaido*, p.48-51 (in Japanese).

Ueki N., and Ikeda Z., 1989: Rearing experiment of newly settled sea cucumber *Stichopus japonicus* using freezed benthic diatoms as food organization. *Bull. Fish. Exp. Station Okayama Pref.*, **7**, 53-55.

Fujiwara A., Yamano K., Ohno K., and Yoshikuni M., 2010: Spawning induced by cubifrin in the Japanese common sea cucumber *Apostichopus japonicus*. *Fish. Sci.*, **76**, 795-801.

Yanagibashi S., Yanagisawa T., and Kawasaki K., 1984: A study on the rearing procedures for the newly settled young of a sea cucumber, *Stichopus japonicus*, with special reference to the supplied food items. *The Aquiculture*, **32(1)**, 6-14 (in Japanese with English abstract).

Yanagibashi S., and Kawasaki K., 1985: Manamako Syubyou Seisan (Seed production of *Stichopus japonicus* Selenka). In: Syouwa 59-nendo Gyoumu Houkoku Aichiken Suisansikenjoh (The 1984 Technical report of Aichi Fisheries Research Institute), p.16-19. Aichi Prefecture, Japan (in Japanese).

水研センター研報，第40号，135－143，平成27年
Bull. Fish. Res. Agen. No. 40, 135－143, 2015

Fluorochrome Marking of Out-planted Green Sea Urchins, *Strongylocentrotus droebachiensis*, for Sea Ranching and Restocking Programs in the Gulf of Maine, USA

Pamelia Fraungruber[*1], Stephen D. Eddy[*1], Nicholas P. Brown[*1], and Larry Harris[*2]

Abstract: Marking calcified structures with fluorochromes is done in a variety of vertebrate and invertebrate species to tag individuals for growth, population, and ecological studies. Here, we describe the use of the fluorochrome tetracycline to identify hatchery reared green sea urchins released on-bottom onto two aquaculture leases known as Job and Sloop, located in the Gulf of Maine, USA. This was done to examine the viability of sea ranching and stock enhancement by looking at recovery rates and growth of reseeded juveniles over the course of two years. 21,000 hatchery reared green sea urchin juveniles *(Strongylocentrotus droebachiensis)* were marked with the fluorochrome tetracycline when they were at 10 – 20 mm test diameter, and released onto 400m^2 study areas located at each lease. Juveniles from the same hatchery cohort were simultaneously reared in a land-based recirculating aquaculture system so that sea ranching could be compared with tank farming. The release areas were surveyed by SCUBA divers at 3-5 month intervals for over two years. Urchins were collected from the field, measured, and dissected to remove the jaw structures, which were then examined with fluorescence microscopy. Tetracycline fluorescence was detected for up to 27 months post-release in recaptured urchins. Numbers of recaptured marked urchins fluctuated over time, causing large variability in population survival estimates for each site at each sample interval. Size measurements of recaptured urchins showed a decline in average test diameter at the Job site, but at the Sloop site average test diameter increased during the two year study. Green sea urchins from the same hatchery cohort reared in a land-based tank system had significantly better growth than those recaptured from either lease site. Environmental factors, rather than genetic factors (hatchery source), were likely the cause of the size differences observed between hatchery seed recaptured from the lease sites and those reared in tank culture. Site factors may have resulted in size dependant mortality and/or out-migration of larger urchins. One of the limitations of the mark/recapture approach with sea urchins is that dive surveys need to expand over time to account for urchin movement away from the release area. Given the high cost of such efforts, this may not be practical or cost effective. Because the marked jaw structures were internally located, it was not possible to identify marked sea urchins in the field, and the animals had to besacrificed for laboratory analysis. Recent advances in fluorochrome marking and visualization could allow field identification of marked urchins. This would enhance the ability of resource managers to evaluate restocking programs in the Gulf of Maine, as well as to assign provenance or ownership of sea ranched urchins.

Key Words: green sea urchins, fluorochromes, sea-ranching

2015年１月30日受理（Received on January 30, 2015）
*1 University of Maine, Center for Cooperative Aquaculture Research, Franklin, Maine, USA
*2 University of New Hampshire, Durham, NH, USA Larry.Harris@unh.edu
Corresponding author: Stephen D. EddyE-mail: steve.eddy@maine.edu

The green sea urchin *Strongylocentrotus droebachiensis* has been an economically important fisheries species in the Gulf of Maine, USA (GOM) since the 1980's. Catch levels peaked in 1993 at 19,050 metric tons, and the fishery value peaked in 1995 at $35,604,275. However, these large annual harvests couldn't be sustained, and ecological changes have contributed to a steep decline in wild stocks (Steneck *et al.*, 2004). Since 2008 the annual catch in Maine has averaged about 1,300 mt, with an average value of about $5.5 million.Although the fishery may not return to 1990 levels, resource managers and fishermen believe that wild stocks can and should be rebuilt to allow for increased harvest levels. Several management approaches have been taken to help rebuild natural stocks, mostly based on fishing restrictions. Stock enhancement through release of hatchery seed has also been discussed, but uncertainty regarding its ecological and economic viability has discouraged public funding of any sustained stock enhancement programs in the GOM.

Evaluating the effectiveness and benefits of sea urchin reseeding programs is an essential but complex task. The economic return will be a function of seed production costs, growth and survival of out-planted seed, and market prices at the time of recapture. In Japan, sea urchin stock enhancement, known as reseeding, has been done at large scale (>50 million seed annually) for over 20 years (Agatsuma *et al.*, 2004; Sakai *et al.*, 2004). In Hokkaido it cost 4-10 JPY (4-8 US cents) to produce one seed of 5 mm test diameter, and nearly 3x that for 20 mm seed (Sakai *et al.*, 2004). The economic benefits of reseeding to the Japanese fishery remain uncertain; in some cases catches have declined or remained static despite widespread reseeding (e.g. *Strongylocentrotus intermedius* in Hokkaido) , whereas in other cases reseeding is correlated with improved catch levels (e.g. *S. nudus* at Esan near Hakodate City) (Agatsuma 2014, in publication). Ultimately, the costs of seed production versus the economic return to the fishery must be considered in the context of cultural values and ecological consequences.

Although increased catch levels might imply that restocking has been successful, it is not evidence of a cause and effect relationship. Ecological changes, increased recruitment, or intensified fishing effort can also lead to improved stocks or increased catch levels. Measures of survival, growth, and return to the fishery are needed to assess the cost/benefit of stock enhancement. This can only be done if released stock can be differentiated from wild stock, but there are no discernible external differences between hatchery and wild urchins (Agatsuma *et al.*, 2004). However, sea urchins can be internally marked with fluorochromes, either through injection or bath immersion. Kobayashi and Taki (1969) were the first to use tetracycline to mark the sea urchin S. *intermedius* for growth studies. Since that time, a variety of studies have used fluorescent markers to identify sea urchins in the lab or in the field (Ellers and Johnson, 2009). This paper describes the use of fluorochrome marking to identify hatchery seed of *S. droebachiensis* released onto ocean bottom leases in the GOM in order to evaluate sea ranching. Sea ranching is similar to reseeding, but in this case the juveniles were released onto privately held aquaculture leases. The project was carried out by the Center for Cooperative Aquaculture Research (CCAR), working with industry partner Friendship International (FI), a sea urchin trading company based in Maine. We were interested in ascertaining whether this privatized mode of reseeding could be a viable model for the fishery. To do this we needed to determine if released seed would remain within lease site boundaries, and whether growth and recovery rates would be sufficient to realize a return to the lease site operator or to the fishery.

Materials and Methods

Hatchery: The CCAR is a multi-species aquaculture research and development facility operated by the University of Maine (http://www.ccar.um.maine.edu/index.html). Hatchery production for the project was carried out at the CCAR in the spring of 2009 (Feb.-June). Green sea urchinswere induced to spawn (N=39 females and 30 males) to provide gametes that were fertilized for larval rearing. Laboratory spawning, fertilization and larval rearing methods for *S. droebachiensis* are similar to those described for many other sea urchin species (McBride, 2005). Larvae were reared in conical bottom 230 L clear

fiberglass vats continuously supplied with fresh seawater at about 12 ℃. *Dunaliella tertiolecta and Rhodomonas salina* were the primary algal feeds. The larvae were competent for settlement at 24-30 days post-fertilization. Following settlement, juveniles were reared for an additional period of 8-10 months in a land based nursery system. During the nursery period they were held in plastic hydroponic plant baskets in shallow fiberglass raceways, and fed *ad libitum* with freshly harvested *Saccharina latissima.*

Fluorochrome marking and visualization: The juveniles were marked (tagged) with tetracycline about four months before release onto the lease sites, using methods adapted from Ellers and Johnson (2009). Juvenile urchins were graded into perforated baskets, and immersed for 24 hours in tanks filled with 0.2 μm filtered seawater and 37.5 mg per L^{-1} tetracycline (Sigma-Aldrich Tetracycline T3258). Urchins were fed to satiation before and during tagging to ensure active growth and uptake of the fluorochrome into the calcareous exoskeleton. Two weeks following marking twenty-five urchins were examined using a fluorescence microscope. The jaws of each individual were removed and placed in a sodium hypochlorite solution to dissolve all organic material, leaving only the calcareous jaws behind. These structures were then examinedthrough a GIB filter using a Zeiss Axio Imager Z1 fluorescence microscope. Oxytetracycline goes through excitation at 390nm and emission at 560nm. Tags appear as a bright line of fluorescence spanning the jaw horizontally, and for the most part were easily identifiable (Fig. 1). 100% of those examined directly after tagging had clearly visible tags.

Sea ranching: The tagged juveniles were released at two aquaculture leases located in Penobscot Bay, Maine. Site 1 (Sloop) was located off of Northaven, Maine near Sloop Island (44° 12.2'N 68° 50.1'W) and Site 2 (Job) was off of Camden, Maine near Job Island (44° 13.5'N 68° 50'W). Each site comprised two acres (0.81 ha) of sea bottom, with a mean water depth of about 2-5 m. The leases were marked with buoys to indicate that harvesting urchins by dragging nets across the bottom was prohibited. In February of 2010, 10,500 juveniles were released at each site onto a small study area located approximately within the middle of each lease. The

Fig. 1. A tetracycline marked green sea urchin *Strongylocentrotus droebachiensis* demipyramid viewed using a WIB filter on a fluorescence compound microscope.

juveniles were transferred in plastic bags by divers onto the bottom and distributed along transect lines laid out to 15 m in all four compass directions, encompassing a total area of 400 m^2. Between 1,000 and 1,500 juveniles were released at 5 m and 10 m markers along the transects to ensure an even distribution. The juveniles were not enclosed and therefore were free to move. No feeding or any other husbandry activity was conducted during the two years following the release.

Site surveys: The sites were characterized in a previous study (Kirchhoff *et al.*, 2008), but prior to out-planting an initial transect dive was done to estimate the extent of existing sea urchins, predators, and bottom cover. At each release area a baseline was laid out in a North-South orientation and five transect lines were laid out on a perpendicular (East-West) bearing extending to 10 m. Sample quadrats consisting of a 1 m2 PVC frame were placed at the 10 m marker in each direction, at the center of the transect, and just over the baseline (0 m on transect), for a total of 15 quadrats per site. During the pre-release survey the bottom substrate was characterized and the numbers of predators (crabs, sea starts, etc.) and naturally occurring (pre-existing) urchins were counted. The extent and composition of algal feed was also observed for each site. The out-planted areas were then dive surveyed on six more occasions at 3-5 month intervals over the course of 27 months. All urchins within each

sample quadrat were enumerated and those between 4-30 mm TD were collected in numbered mesh tubes to be taken to the laboratory for measurement and identification (absence/presence of fluorochrome marker). Urchins smaller or much larger than the original release size were not collected in early surveys, but during later surveys larger urchins were collected to account for any growth.

Tank culture: During the two year sea ranching study 9,500 green sea urchins from the same hatchery cohort as the lease site urchins were reared in a tank system at the CCAR, to compare growth and survival of lease site urchins with juveniles reared on land. The juveniles were stocked into raceways assembled to form a slanted V interior profile (V-trough), with a perforated bottom plate to remove wastes. The V-troughs were plumbed into a recirculating seawater aquaculture system equipped with a parabolic filter for solids removal, moving bed biofilter, foam fractionator, oxygen injection, 3 hp chiller, and UV sterilizer. Rearing temperatures were held between 6-16 ℃ year round and the juveniles were fed high quality formulated diets (Nofima diet from Norway). It was anticipated that sea urchins reared under these conditions would have good growth and survival, to provide a benchmark by which the lease site urchins could be compared.

Specimen analysis: Specimen bags containing urchins from the sample quadrats were brought back to the lab, drained and frozen until analysis. These were later (within 2-6 weeks) thawed in seawater, and all individuals were blotted dry and weighed to the nearest 0.1g. Test diameter (TD) was measured to the nearest 0.1mm with digital calipers (model CD-6PMX Mitutoyo Corporation, Kawasaki, Japan). Each sea urchin collected from the lease sites was analyzed for the presence of oxytetracycline marking, as described above. In some cases multiple or single bands of auto-fluorescence were seen that appeared atypical or ambiguous (e.g. diffuse). Sources of ambiguity and therefore error in identification included size of the jaws, intensity of the light used to make the tags fluoresce, and ambient light from the surrounding room. If the results were uncertain, then the jaws were either reevaluated or marked as "untagged". Urchins that were clearly tagged were considered as recaptured (hatchery origin).

Urchins reared in the land-based tank system were sampled at intervals coinciding with the lease site surveys. Thirty urchins from each tank were randomly removed and measured for weight to the nearest 0.1 g, and TD to the nearest 0.1 mm using digital calipers.

Data analysis: The average number of total urchins (tagged and untagged) per square meter was calculated for each study area and survey date as the total number of urchins collected per site divided by the number of sample quadrats (usually 15). The number of released seed remaining at each site and survey date was estimated as the average number of recaptured (tagged) urchins per sample quadrat (m^2) x 400 m^2 (the size of the release area as a whole). The mean, minimum and maximum test diameter of recaptured urchins was calculated for each site and survey date. Chi squared tests were used to determine whether or not the numbers of tagged and untagged urchins were significantly different from each other. The standard deviation of the mean test diameter was determined to see if the average size of recaptured urchins significantly differed (±1 SD) between the two sites and from the tank reared urchins. Data were plotted to display trends in numbers, average TD, and maximum TD of recaptured urchins at each site over time, and the TD of lease site urchins was compared with that of tank reared urchins.

Results

Site characteristics: The two sites were less than six nautical miles apart and of comparable depth (2-6 m mean water), but they differed in terms of exposure, current, bottom substrate, and population density of naturally occurring (pre-existing) urchins. The Job Island site had relatively uniform depth, but was subject to periods of extreme slack tide and periods of strong current. The bottom substrate at Job was 80% rock cobble with several small boulders throughout, which were populated with macroalgae, but relatively little drift algae was found. Predators were not found in abundance, with only one large Jonah crab (*Cancer borealis*) observed, and the initial population density of pre-existing urchins at the Job site was 2.25 animals/m^2. At the Sloop site,

the bottom substrate was 80% shell hash, which provided abundant refuges for small and medium-sized urchins. The study area was on a sloped ledge, so the depth varied across the area compared with Job, which was more flat. A few small boulders were found on the Sloop site with macroalgae growing on them, and drift algae, mostly kelp, were abundant. The Sloop Island site had an abundance of large urchins and sea stars present on it at out-planting. The initial population density of pre-existing urchins at the Sloop site was 4.5 animals/m^2.

Recapture rates: Sea urchins were found on both study areas at every survey for over two years. The total number (tagged and untagged) found at each survey ranged from 4 – 674 at the Job site and from 194 – 397 at the Sloop site. Tagged urchins (hatchery origin) were recaptured at both sites and at every dive survey up to the last, 27 months post-release. Recapture rates declined in the first year but then significantly spiked in the summer of the following year at both sites, before again declining in subsequent surveys (Fig. 2). At the Job site 10% to 100% of the urchins collected during each dive survey were determined to be of hatchery origin, and at Sloop 35% to 71% of collected urchins were of hatchery origin. It's important to note that on the one occasion when 100% of the animals collected at Job were tagged, the entire sample population consisted of just four animals, all very small (<7 mm TD). At the final survey a total of 107 urchins were collected from Job and about 30% of these were tagged. At the final Sloop survey the urchin population showed a significant decline from previous levels, and there was evidence (disturbed grounds, gear tracks, and broken tests) that the site had been recently fished by a dragger boat.

Population estimates: Population estimates of hatchery origin urchins remaining within the 400m^2 release areas at each survey varied in direct proportion with the recapture rates (Fig. 3). Originally, 10,500 urchins were released at each study area. Extrapolation from dive surveys indicated that the number of hatchery origin urchins remaining within the Job release area at each surveyranged from 45 to 36,894; with 3,306 projected as still remaining at 27 months post-release. At the Sloop site, population estimates of hatchery urchins remaining at each survey ranged from 3,680 to 18,165; with 7,360 projected as still remaining at the final survey, 27 months post-release (Fig. 3).

Average and maximum size: The average test diameter (TD) of hatchery origin urchins recaptured at the Job site declined to 5.1 mm over the course of the study, which was the minimal release size, but TD increased at the Sloop site (Fig. 4). At Job, the average TD declined from 10.6 mm at release to 5.1 mm 27 months post-release, whereas at Sloop the average TD increased from 11.3 mm at release to 18.3 mm at 27 months. The largest marked urchin recaptured from any of the surveys at Job during the course of the study was 19.7 mm (Aug 2010), and at Sloop it was 49.3 mm TD (Sept 2011, 19 months post-release) (Fig. 5). The Job site had a disproportionate number of small urchins remaining on it at every survey throughout the course of the

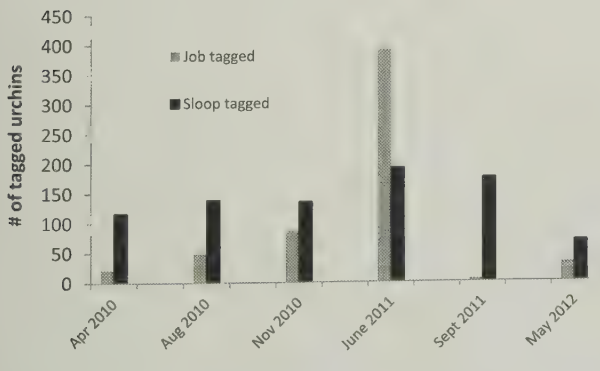


Fig. 2. Total numbers of hatchery origin *S. droebachiensis* recapturedfrom two release sites in Penobscot Bay, Maine at each survey.

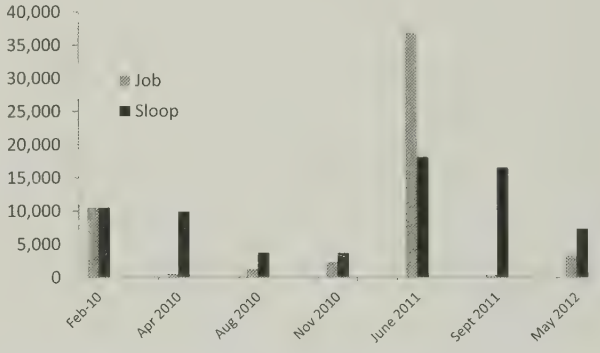


Fig. 3. Estimated population of hatchery origin *S. droebachiensis* remaining within the study area at each lease site and sample date. Calculated as average number of recaptured urchins per sample quadrat (m2) x 400 m2 (total study area).

Fig. 4. Average test diameter of hatchery origin *S. droebachiensis* recaptured at two release sites in Penobscot Bay, Maine at each dive survey, and in tank culture at the CCAR. Error bars = ±1 standard deviation from the mean.

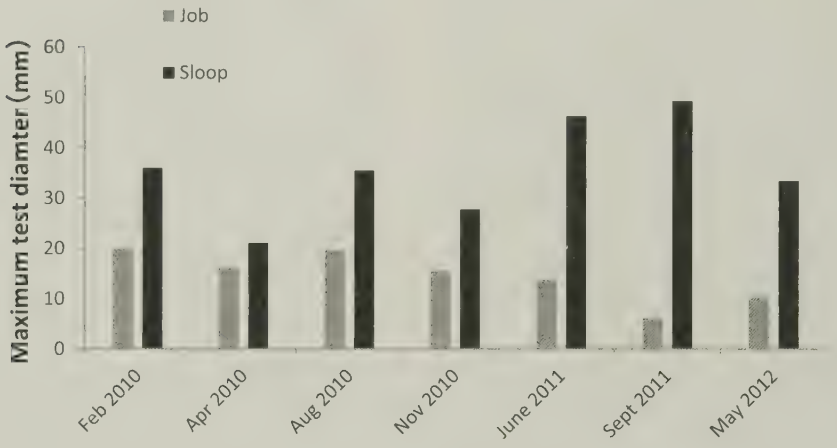


Fig. 5. Maximum test diameter of hatchery origin *S. droebachiensis* recaptured at two release sites in Penobscot Bay, Maine at each dive survey.

study. Of the total number of urchins (sum of six surveys) recaptured from Job, 84% were ≤6mm. At the Sloop Island site, only 1% of the total recaptured urchins were ≤6mm.

Growth rates diverged between land and sea based hatchery urchins within the first year of the study. Sea urchins reared in the land-based culture system were much larger on average at the end of the two year study than those recaptured from either ocean lease site (Fig. 4). After 27 months the largest urchin sampled in the tank culture system was 53.4 mm, and ≈ 1/3 of the tank reared urchins were ≥40 mm.

Discussion

Few previous studies in North America have monitored survival and growth of tagged sea urchins released into the field. Dumont *et al.* (2004) released three size groups of green sea urchins tagged with tetracycline onto a small study area. Similar to the present study, they found that recapture rates were size and time specific: 69% for <10 mm and 2% for >15 mm urchins after nine days, and 25% and 0% respectively after forty days. In a study by Rogers-Bennett *et al.* (1994), red urchin juveniles (*Strongylocentrotus franciscanus*) were tagged and

released onto study areas that varied in depth. Recovery rates after 12 months were 21% from shallow habitats and 11% from deep habitats.

The present study provides evidence that hatchery reared green sea urchins can be successfully out-planted for reseeding or sea ranching in the Gulf of Maine. Success is defined as the ability of seed to survive and grow to legal harvest size (52 mm) within 5 years of release. We saw that released juveniles survived and remained for an extended period (27 months) within each release area. However, recaptured juveniles were disproportionately smaller at one site (Job) than at the other (Sloop). This suggests that site factors modified the size distribution of surviving or remaining out-plants in different ways at the two sites. The Sloop Island site may have had a habitat more favorable for sea urchins, with more and larger refuges, and greater feed abundance.

Following settlement, juvenile green sea urchins take refuge under rocks, in crevices, or under debris as an adaptation to escape predation (Cameron and Schroeter, 1980; Dumont *et al.*, 2004). Here they graze on diatoms, coralline algae and detritus (Raymond and Scheibling, 1987). While both sites in the present study supported urchins, the shell hash at the Sloop Island site was full of cracks, holes and larger spaces, providing refuges for a broader size range of juveniles. The rock cobble at the Job Island site was, for the most part, flat against the sediment, with fewer and smaller hiding places for juveniles. The rock cobble had small interstices that were well suited for juveniles at or below 5–6 mm, but too small for larger urchins. Most of the urchins recovered from the Job site surveys, whether wild or tagged, were < 15 mm TD.

The Job site generally had lower recapture rates of tagged seed than the Sloop site, indicating that it was less hospitable for out-planted sea urchins. The notable exception occurred in the summer of the second year, when a large and significant number of small urchins were captured at the June 2011 Job site survey, and subsequently identified as hatchery origin due to presence of the fluorochrome mark. This spike in recapture numbers could have been due to misidentification (e.g. detecting auto or pseudo-fluorescence and attributing it to the tag), or it could have been a sampling artifact. Presumably, misidentification would have occurred equally at Sloop at this survey date, and it did not. Although Sloop had higher recapture numbers (per quadrat and total) at this survey date than at other surveys, they were not significantly different from the other Sloop surveys. Also, we were concerned about this issue and any specimens with atypical fluorescence patterns were considered as unmarked. For these reasons, we believe that the spike in recapture numbers observed at Job during the June 2011 survey was a sample artifact. Every sample quadrat had to be thoroughly and equally searched, often by overturning rocks and shells to find hidden urchins. This effort had to be consistent between sites and survey dates, which in practice was difficult to accomplish. Under varying field conditions of bottom substrate, current, turbidity, ambient light, and temperature, it's likely that the success rate for finding urchins would vary between sites and dates. In addition, random movement patterns of urchins onto and off of the study areas probably occurred, because urchins move in response to food availability and the presence/absence of predators (Dumont *et al.*, 2007). At about 15mm TD sea urchin juveniles are less vulnerable to predation, and a shift from cryptic to active foraging occurs (Dumont *et al.*, 2004). Migration of urchins larger than 10 mm TD away from the release area in search of feed or refuge might explain the disproportionate numbers of small tagged urchins seen at the Job site.

Active foraging enhances the availability and quality of macroalgae, increasing the growth rate. When there is abundant food sea urchins will aggregate in high densities, and they can remain stationary for several months or longer (Dumont *et al.*, 2007). In the present study, both sites provided feed in the form of encrusting algae and particulate macro-algae. However, the Sloop site was more exposed and had greater currents (Kirchhoff *et al.*, 2008), and urchins at this site thus had access to large pieces of drift algae, mostly kelp, that were carried onto the site by the current. This greater feed availability might explain why recaptured urchins had a larger average and maximum TD at Sloop than at Job. The lack of a substantial food source at the Job site might have encouraged

more of the larger urchins to leave the site, while also causing slow growth of the small urchins that remained, due to low food intake. Green sea urchin growth rates can be highly variable in the natural environment, primarily in response to feed availability and type (Nestler and Harris, 1994; Brady and Scheibling, 2006). Growth can be very slow and rates of ≤ 0.25 mm per year have been documented for urchins found in tide pools (Russell, 1998). We observed that green sea urchins from the same hatchery cohort reared in the land-based culture system had significantly better growth than those recaptured at either lease site. This is further evidence that growth potential at the lease sites was limited more by environmental factors than by genetics or by the fact that the urchins were of hatchery origin.

In the present study we were able to differentiate hatchery origin from wild urchins for up to 27 months in the field. Johnson *et al.* (2013) reported that tetracycline fluorescence could be detected for at least two years in green sea urchins held in the lab, when tetracycline was administered via injection. The fact that fluorochromes can persist for such extended periods makes this marking/tagging method invaluable for long term lab and field studies of sea urchins, and was essential to carrying out the research described above. Recent advances in the application and visualization of fluorochromes offer further advantages, which could bring down costs and improve the effectiveness of sea urchin mark/recapture studies. Ellers and Johnson (2009) describe methods to create multiple marks on the demipyramids (e.g. at intervals or with multiple fluorochromes), which would allow for differentiation of multiple year classes released into the field. The same authors also describe visualization of fluorochromes on external structures such as the skeletal plates (test) and spines, which allows tagged individuals to be identified without sacrifice (Johnson *et al.*, 2013). Ultimately, development of a field portable device for visualizing fluorochromes seems feasible, to allow reliable identification of stocks *in situ* while minimizing adverse impact on the population (Johnson *et al.*, 2013). These methods provide powerful tools for evaluating the results of future restocking and sea ranching programs for green sea urchins in the Gulf of Maine.

References

Agatsuma Y., Sakai Y., and Andrew N. L., 2004: Enhancement of Japan's sea urchin fisheries. *In*: J. M. Lawrence and O. Guzman (eds.) Sea Urchins: Fisheries and Ecology. DEStech Publications, Inc., Lancaster. pp. 18–36.

Agatsuma Y., 2014: Reseeding of sea urchins in Japan. *In*: Brown N. P., and Eddy S. D. (eds) Aquaculture of Echinoderms, *In press* John Wiley and Sons, New York, NY

Brady S. M. and Scheibling R. E., 2006: Changes in growth and reproduction of green sea urchins, *Strongylocentrotus droebachiensis*, during repopulation of the shallow subtidal zone after mass mortality. *J. Exp. Mar. Bio. Ecol.*, **335**, 277–291.

Cameron R. A., and Schroeter S. C., 1980: Sea urchin recruitment: Effect of substrate selection on juvenile distribution. *Mar. Ecol. Prog. Ser.*, **2**, 243–247.

Dumont C., Himmelman J. H., and Russell M. P., 2004: Size-specific movement of green sea urchins *Strongylocentrotus droebachiensis* on urchin barrens in Eastern Canada. *Mar. Ecol. Prog. Ser.*, **276**, 93–101.

Dumont C. P., Himmelman J. H., and Robinson S. M., 2007: Random movement pattern of the sea urchin *Strongylocentrotus droebachiensis. J. Exp. Mar. Bio. Ecol.*, **340**, 80–89.

Ellers O., and Johnson A. S., 2009: Polyfluorochrome marking slows growth only during the marking month in the green sea urchin *Strongylocentrotus droebachiensis. Invert. Biol.*, **128**, 126–144.

Johnson A. S., Salyers J. M., Alcorn N. J., Ellers O., and Allen J. D., 2013: Externally visiblefluorochrome marks and allometrics of growing sea urchins. *Invert. Biol.*, **132(3)**, 251–269.

Kirchhoff N., Eddy S., Harris L., and Brown N., 2008: Nursery-phase culture of green seaurchin *Strongylocentrotus droebachiensis* using "on-bottom" cages. *J. Shellfish Res.*, **27(4)**, 921–927.

Kobayashi S., and Taki J., 1969: Calcification in

sea urchins. A tetracycline investigation of growth of the mature test in *Strongylocentrotus intermedius*. *Calcif. Tissue Res.*, **4**, 210-223.

McBride S., 2005: Sea urchin aquaculture. *Amer. Fish. Soc. Symp.*, **46**, 179-208.

Nestler E. C., and Harris L. G., 1994: The importance of omnivory in *Strongylocentrotusdroebachiensis* (Muller) in the Gulf of Maine. In: B. David (ed.) Proceedings of the 8th International Echinoderm Conference, Dijon, France. A. A. Balkema, New York. pp. 813-818.

Raymond B. G., and Scheibling R. E., 1987: Recruitment and growth of the sea urchin *Strongylocentrotus droebachiensis* (Muller) following mass mortalities off Nova Scotia, *Can. J. Exp. Mar. Biol. Ecol.*, **108**: 31-54.

Rogers-Bennett L., Fastenau H. C., Hibbard-Robbins T., Kain Z., and Dewees C. M., 1994: Culturing red sea urchins for experimental out planting in northern California. Final Report. Contract FG-2230-MR., Bodega Marine Laboratory, California. pp. 1-10.

Russell M. P., 1998: Resource allocation plasticity in sea urchins: rapid, diet induced, phenotypic changes in the green sea urchin, *Strongylocentrotus droebachiensis*. *J. Exp. Mar. Biol. Ecol.*, **220**, 1-14.

Sakai Y., Tajima K. I., and Agatsuma Y., 2004: Mass production of seed of the Japanese edible sea urchins *Strongylocentrotus intermedius* and *Strongylocentrotus nudus*. *In*: J. M.Lawrence and O. Guzman (eds.) Sea Urchins: Fisheries and Ecology. DEStech Publications, Inc., Lancaster. pp. 287-298.

Steneck R. S., Vavrinec J., and Leland A. V., 2004: Accelerating trophic-level dysfunction in kelp forest ecosystems of the western North Atlantic. *Ecosystems*, **7**, 323-332.

水研センター研報，第40号，145－153，平成27年
Bull. Fish. Res. Agen. No. 40, 145－153, 2015

Improving the Food Quality of Sea Urchins Collected from Barren Grounds by Short-Term Aquaculture under Controlled Temperature

Tatsuya UNUMA[*1], Yuko MURATA[*2], Natsuki HASEGAWA[*1], Sayumi SAWAGUCHI[*1], and Kazuhiro TAKAHASHI[*3]

Abstract: In west Hokkaido, there are many barren grounds where *Mesocentrotus nudus* is abundant. These sea urchins have poor commercial value due to their thin gonads, but can become marketable by intensive feeding for gonad enhancement. In general, the quality of sea urchin gonads as food products decreases as gametogenesis progresses. Mature ovaries and testes are not suitable as food products because of the unpleasant taste caused by gamete content and the melting appearance caused by gamete flow via breakage of the gonoduct. Immature to pre-mature gonads that contain predominantly nutritive phagocytes (somatic nutrient storage cells) and not copious gametes have a higher commercial value. Thus, enhancing the nutrient accumulation into nutritive phagocytes plus suppressing gametogenesis is advantageous for sea urchin aquaculture. We are developing short-term aquaculture techniques to improve the food quality of *M. nudus* collected from barren grounds under controlled temperature to suppress gametogenesis. Rearing *M. nudus* under a low temperature between summer and autumn has proved to be effective to increase the gonad size without the quality deterioration caused by maturation.

Keywords: aquaculture, food quality, gametogenesis, gonad, sea urchin, temperature

For decades, the loss of seaweed beds and the expansion of barren grounds has been a serious problem for coastal fisheries in Japan (Fujita, 2010). The bare grounds populated by sea urchins are known as urchin barrens (Pearse, 2006; Fujita *et al.*, 2008). In west Hokkaido, there are many urchin barrens, where *Mesocentrotus nudus* is abundant (Fig. 1). The sea urchins in these barrens have poor commercial value because they have thin gonads (edible portion) due to deficiency of their main food, macroalgae. However, they can become marketable by intensive feeding for gonad enhancement in short-term aquaculture (Agatsuma and Nishikiori, 1991; Unuma and Kayaba, 2015). If the cultured *M. nudus* of improved quality are sold in autumn, out of the fishing season of this species, higher market price can be achieved.

Unlike the gonads of other animals, sea urchin

Fig. 1. An urchin barren in Suttsu, west Hokkaido. The bare ground is populated by *Mesocentrotus nudus* that has poor commercial value because of thin gonads. The photograph was taken by Kazuhiro Takahashi.

2015年1月30日受理（Received on January 30, 2015）
*1 Hokkaido National Fisheries Research Institute, Fisheries Research Agency, Kushiro, Hokkaido 085-0802, Japan
E-mail: unuma@fra.affrc.go.jp
*2 National Research Institute of Fisheries Science, Fisheries Research Agency, Fukuura, Yokohama 236-8648, Japan
*3 Mariculture Fisheries Research Institute, Hokkaido Research Organization, Muroran, Hokkaido 051-0013, Japan

ovary and testis (both of which are equally preferred as food) play a role as a nutrient storage organ (Walker, 1982; Walker *et al.*, 2013; Unuma, 2002, 2015). A thorough understanding of the unique characteristics of sea urchin reproduction should permit novel methods to improve the quality of gonads as food products in its aquaculture. We are currently conducting research and development for short-term aquaculture techniques to maximize the value of *M. nudus* collected from urchin barrens under controlled temperature and harvest them when wild *M. nudus* is scarce in the market. In this paper, we describe the theoretical background to improve the food quality of *M. nudus* by manipulating environmental conditions and give a brief overview of our ongoing research.

Features of Gametogenesis

The sea urchin has five gonads attached internally to the test (shell). A lobe of gonad consists of hundreds of gonadal acini and resembles a bunch of grapes (Fig. 2A). There are two major populations of cells inside the acinus (Fig. 2B): germ cells (GCs, from oogonia to ova in the ovary and from spermatogonia to fully differentiated spermatozoa in the testis) and somatic cells called nutritive phagocytes (NPs) that are present in both sexes (Walker, 1982; Walker *et al.*, 2013). NPs store nutrients necessary for gametogenesis and supply it to GCs (Walker, 1982; Unuma, 2002).

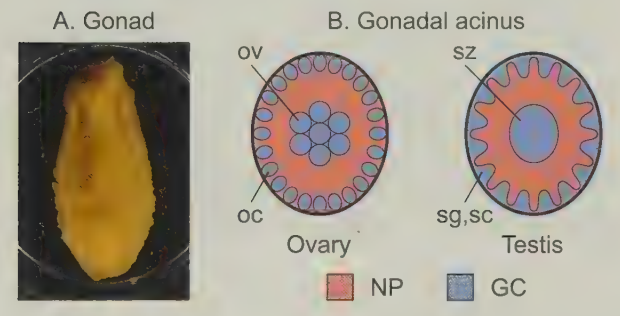


Fig. 2. Structure of sea urchin gonad. (A) A gonad of *Mesocentrotus nudus*. A lobe of gonad consists of hundreds of acini. (B) Schematic drawing of the inside of gonadal acinus. Each acinus contains two main types of cell populations: germ cells (GCs) and nutritive phagocytes (NPs). NPs are somatic cells that store nutrient necessary for gametogenesis.
oc = oocyte, ov = ripe ovum, sc = spermatocyte, sg = spermatogonium, and sz = spermatozoon.

During the annual reproductive cycle, gonads of both sea urchin sexes pass through a predictable series of structural changes (Unuma and Walker, 2009; Walker *et al.*, 2013). Fig. 3 shows histological changes in the ovary and testis of *M. nudus* during gametogenesis as classified into five stages by Fuji (1960a) with modifications (Unuma, 2002).

Stage 1. Immature gonad before gametogenesis: In both sexes each acinus is filled with NPs (eosinophilic cell populations). In ovaries, a few young oocytes are present at the periphery of the acini. Hematoxylin-stained round spots, residue from phagocytized ova (Masuda and Dan, 1977; Tominaga and Takashima, 1987), are occasionally present centrally in the ovarian lumen. In testes, detection of spermatogenic cells is sometimes difficult at this stage in paraffin sections. Instead, many hematoxylin-stained speckles, residue from phagocytized spermatozoa (Kato and Ishikawa, 1982; Reunov *et al.*, 2004), are often present in NPs. These speckles, which are amorphous unlike the round spots observed in the immature ovary, are a useful feature to distinguish testes from ovaries.

Stage 2. Beginning of gametogenesis: Many developing oocytes or clusters of spermatogonia are present at the periphery of the acini, and the gonadal lumina are still filled with NPs.

Stage 3. Middle of gametogenesis: NPs are replaced with ripe ova or spermatozoa in the center of the gonadal lumina. Numerous developing oocytes or clusters of spermatogonia and spermatocytes (Ward and Nishioka, 1993; Walker *et al.*, 2005) are present at the periphery of the acini. NPs are gradually decreasing in size and are present between the GCs.

Stage 4. Fully mature gonads at the end of gametogenesis: The gonadal lumina are filled with ripe ova or spermatozoa. Shrunken NPs, which have already lost nutrients, are present only at the periphery of the acini.

Stage 5. After spawning: The gonadal lumina have numerous empty spaces and a few residual ova or spermatozoa. NPs gradually phagocytize residual gametes and begin to grow as they store nutrients. After this stage, gonads return to Stage 1 and a new cycle starts.

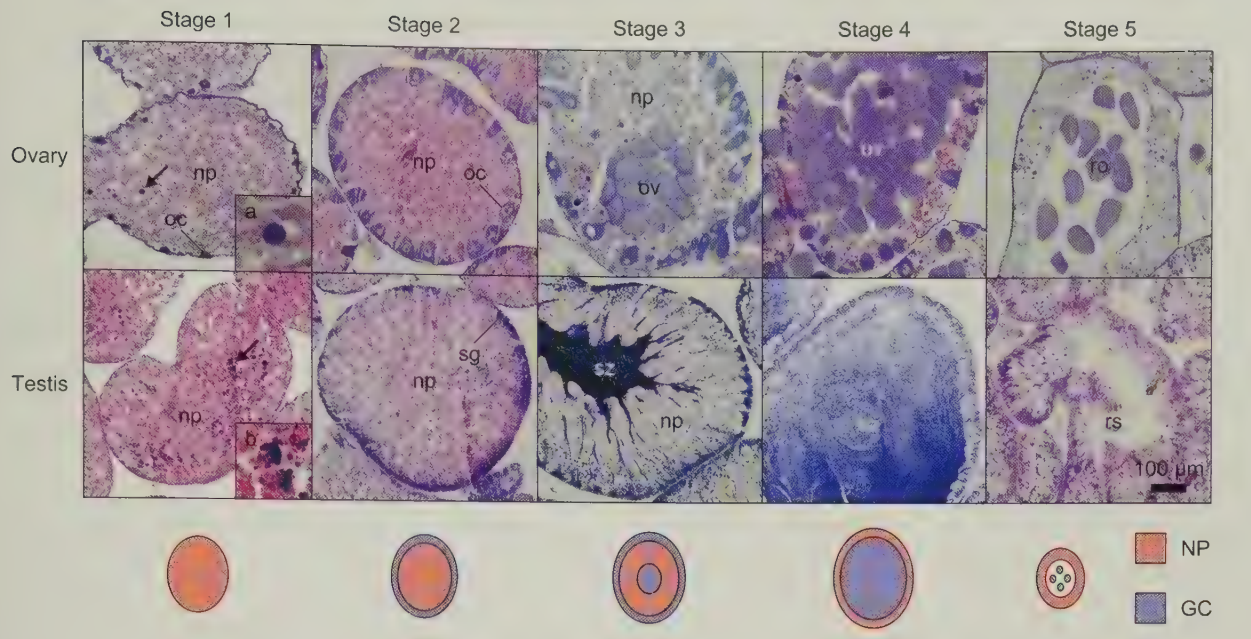


Fig. 3. Histological changes in the ovary (upper panels) and testis (lower panels) of *Mesocentrotus nudus* during gametogenesis. Paraffin-embedded sections are stained with hematoxylin and eosin. Schematic drawing below the photomicrographs shows structural features that are common between the ovarian and testicular acini. At stage 1, the gonadal lumina are filled with nutritive phagocytes. At stage 2, many developing oocytes or clusters of spermatogonia are present at the periphery. At stage 3, nutritive phagocytes are replaced with ripe ova or spermatozoa in the center of the lumina. At stage 4, the lumina are filled with ripe ova or spermatozoa. At stage 5, the lumina contain a few residual ova or spermatozoa. np = nutritive phagocyte, oc = oocyte, ov = ripe ovum, ro = residual ovum, sg = spermatogonium, sz = spermatozoon, and rs = residual spermatozoon. Inset a, round spot derived from a phagocytized residual ovum. Inset b, amorphous speckles derived from phagocytized residual spermatozoa. Scale bar represents 100 μm.

Seasonal Changes in Gonad Size and GCs/NPs Proportion

Sea urchin gonads grow in size not only because gametogenesis increases the size or numbers of GCs but also because NPs store extensive nutrient reserves before gametogenesis (Unuma and Walker, 2009; Walker *et al.*, 2013). Fig. 4 shows the seasonal changes in the gonad index (the ratio of gonad weight to total body weight) and in the proportion of GCs and NPs in *M. nudus*. This species spawns around October and gonad indices rapidly decrease (Fuji, 1960b). After spawning, the gonad index gradually increases until the next spawning. The increase before gametogenesis is attributable to the growth of NPs. NPs accumulate various nutrients, such as proteins (principally the major yolk protein; Unuma *et al.*, 2003, 2011), lipids, and carbohydrates (principally glycogen; Marsh *et al.*, 2013) and increase in size. Proliferation and development of GCs begin

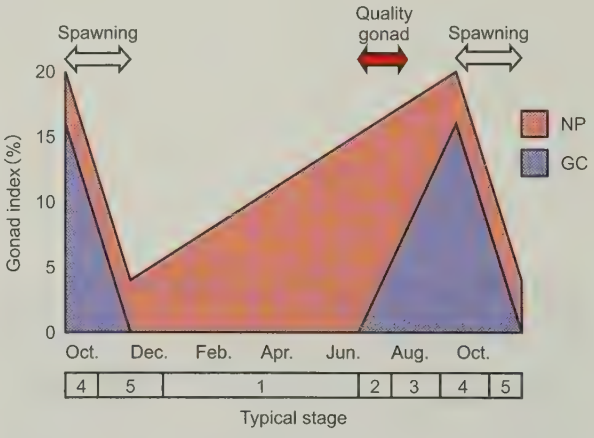


Fig. 4. Diagrammatic representation of the seasonal changes in the gonad index and in the proportion of nutritive phagocytes (NPs) and germ cells (GCs) in *Mesocentrotus nudus*. Typical gametogenic stages are indicated below the graph. Spawning occurs around October. The long-term increase in the gonad index before gametogenesis is attributable to the growth of NPs. The best season for eating gonad is around July.

three months before spawning in *M. nudus* (Unuma, 2009). After gametogenesis begins, the size of the gonad continues to increase but the proportion of NPs in the gonad rapidly decreases.

The Best Season for Eating Gonads

The best season for eating *M. nudus* gonads is around July, when NPs have grown sufficiently but the proportion of GCs is still smaller (Unuma, 2009). Their gametogenic stage is usually from stage 2 to early stage 3 in July (our unpublished observation). Before this period, the size of the gonad is too small and the color of the gonad is not attractive (brownish). After this period, the proportion of GCs becomes higher and the quality as food gradually deteriorates. The most serious problem for food quality is the melting appearance caused by the flow of gametes (Unuma, 2002; Unuma and Walker, 2009). After ripe gonads are taken out of the test, eggs or sperm flow out from the breakage of gonoduct, and the gonads cannot maintain their consistency (Fig. 5). This phenomenon considerably reduces the commercial value of the gonads. Additionally, the gametes cause unpleasant tastes such as bitterness in the mature gonads (Unuma, 2002; Unuma and Walker, 2009).

Relationship between the gametogenic stage of gonads and the food quality varies among species. Ovaries of *Pseudocentrotus depressu*s and *Hemicentrotus pulcherrimus* produce an extremely bitter taste after oogenesis begin (Murata *et al.*, 2002; Murata 2009, Unuma and Walker, 2009). In these species, the season for eating is limited only when their gonads are at stage 1 (Murata 2009; Unuma 2002, 2009). On the other hand, other Japanese sea urchins including *M. nudus* can be eaten until when their gonads are at stage 3, because the maturing gonads do not produce a strong bitterness (Murata 2009; Unuma 2009). In every species, the best season for eating gonads is rather short because of the problems caused by gametogenesis. Low-quality or small gonads can be obtained for a longer period of time, but superior-quality and sizable ones can be harvested for only about one or two months (Unuma, 2002).

Fig. 5. Gamete flow from the mature ovary (left) and testis (right) of *Mesocentrotus nudus*. After mature gonads are taken out of the test, they appear to be melting because of flowing eggs or sperm from the breakage of gonoduct.

Improving Food Quality by Short-term Aquaculture

As we have seen, NPs are more important as food than GCs. Thus, when sea urchins are cultured for gonad enhancement, not GCs but NPs should be increased in the gonads. The sea urchins dwelling at urchin barrens cannot store much nutrient in the gonads due to deficiency of food (Agatsuma and Nishikiori, 1991). However, the sea urchins produce appropriate amount of gametes and spawn even if the food availability is low. Therefore, the schema of gonad index and GCs/NPs proportion (Fig. 4) is turned to be depressed in the sea urchins at urchin barrens (Fig. 6A). We are trying to change this schema by short-term aquaculture as shown in Fig. 6B. The aims of the culture are (1) to promote nutrient accumulation into NPs by intensive feeding and (2) to suppress proliferation and development of GCs by manipulating environmental conditions. Combination of these two aims should prolong the season for quality gonad until the spawning season when wild *M. nudus* is not fished due to quality deterioration as well as for resource management.

Environmental Factors Regulating Gametogenesis

Gametogenic cycles of sea urchins are regulated by environmental factors, such as photoperiod and water temperature. There have been many reports describing the effects of these factors on gametogenesis of sea urchins as listed in Table 1. Most of these researches were conducted as basic biology to identify factors affecting maturation, or as applied biology to develop techniques for promoting

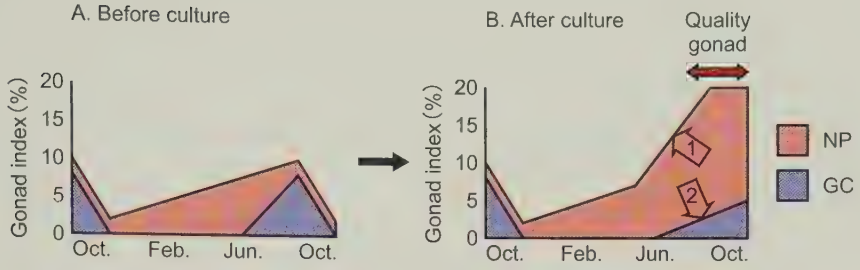


Fig. 6. Purpose of the short-term aquaculture of *Mesocentrotus nudus* collected from urchin barrens. Schema of gonad index and GCs/NPs proportion can be changed from (A) to (B) by rearing under optimized conditions. The aims of the culture are (1) to increase the nutritive phagocytes (NPs) by intensive feeding and (2) to decrease the germ cells (GCs) by suppressing gametogenesis. Combination of these two aims should prolong the season for quality gonads.

Table 1. Researches on environmental factors affecting sea urchin gametogenesis

Factors investigated	Species	literature
Photoperiod	*Eucidaris tribuloides*	McClintock and Watts (1990)
	Paracentrotus lividus	McCarron *et al.* (2010)
	Strongylocentrotus droebachiensis	Walker and Lesser (1998)
		Böttger *et al.* (2006)*
		Dumont *et al.* (2006)
		Kirchhoff *et al.* (2010)
	Strongylocentrotus purpuratus	Cochran and Engelmann (1975)
		Pearse *et al.* (1986)
		Bay-Schmith and Pearse (1987)
Temperature	*Heliocidaris crassispina*	Sakairi *et al.* (1989)
	Hemicentrotus pulcherrimus	Yamamoto *et al.* (1988)
		Ito *et al.* (1989)
		Sakairi *et al.* (1989)
	Pseudocentrotus depressus	Yamamoto *et al.* (1988)
		Masaki and Kawahara (1995)
		Noguchi *et al.* (1995)
	Strongylocentrotus droebachiensis	Garrido and Barber (2001)
	Strongylocentrotus intermedius	Kayaba *et al.* (2012)*
Photoperiod and Temperature	*Paracentrotus lividus*	Spirlet *et al.* (2000)*
		Shpigel *et al.* (2004)*
	Psammechinus miliaris	Kelly (2001)

*Conducted from a view point of suppressing gametogenesis

maturation to obtain gametes out of the spawning season for laboratory use or for seed production. For example, in some sea urchin hatcheries in Japan, out-of-season maturation of brood stock of *Pseudocentrotus depressus*, *Hemicentrotus pulcherrimus* and *Strongylocentrotus intermedius* is promoted by manipulating water temperature (Ito *et al.*, 1989; Masaki and Kawahara, 1995; Noguchi *et al.*, 1995; Sakai, 2015). However, for culturing adult sea urchins to enhance the gonads, not promotion but suppression of gametogenesis is required. To our knowledge, only four studies have been conducted from a view point of suppressing gametogenesis to obtain the gonads of better food quality. In *Strongylocentrotus droebachiensis* (Böttger *et al.*, 2006), *Strongylocentrotus intermedius* (Kayaba *et*

al., 2012) and *Paracentrotus lividus* (Spirlet *et al.*, 2000; Shpigel *et al.*, 2004), maturation was delayed by manipulation of photoperiod, water temperature and both of them, respectively. However, the control regime of environmental conditions for culturing *M. nudus* is still to be clarified, because optimum conditions to suppress gametogenesis effectively varies among different species.

Overview of Our Research

Our final goal is to develop a land-based aquaculture system, using *M. nudus* collected from urchin barrens, controlling their gametogenic cycle, and harvesting high-quality urchins when their wild counterparts are scarce in the fish market. As an initial step, we investigated the effects of water temperature on the size and quality of the gonads in *M. nudus*.

Adult *M. nudus* collected from an urchin barren in Suttsu, west Hokkaido (Fig. 1), were reared from late June to mid October in three tanks maintained at either a constant temperature (10℃ or 15℃) or a variable temperature, similar to their natural environment (control group; 18.0℃ to 22.5℃). They were given a surplus of kelp (*Saccharina longissima*). Over the experimental period, gonad indices increased in all treatments from 7% initially to over 20% after three months. Histological observations revealed that after three months, there were no urchins with fully mature gonads in the 10℃ group, but 6% in the 15℃ group, and 39% in the control group. Sensory tests of final gonads found that the quality of the control group was inferior to that of the 10℃ and 15℃ groups, in terms of both appearance and taste. We conclude that rearing *M. nudus* under a low temperature between summer and autumn is effective to increase the gonad size without the quality deterioration caused by maturation. These results are being prepared for publication in the journal. We are further investigating the relationship between the rearing conditions and the size and quality of the gonads to determine the optimum control regime for culturing *M. nudus*.

Methods to Decrease Water Temperature

To put our findings into practice, economic methods for decreasing the water temperature in aquaculture farms are required. Using electric cooling devices is a classic method but has high energy costs. It would not be practical to use it in a conventional flow-through system. However, closed or semi-closed recirculating system may minimize the problem of energy costs in electric cooling devices.

Deep seawater drawn from a depth of over 300 m has a very low temperature (Nakasone and Akeda, 2000). Large initial investment costs are required to build a facility to draw the water. However, more than ten facilities capable of drawing deep seawater have been constructed in Japan, where it is used for aquaculture, handling of captured fishes, the food industry, medical treatment, and agriculture (Nakasone and Akeda, 2000; Kayaba *et al.*, 2012); including two in west Hokkaido. Therefore, the use of existing facilities is a practical way to adopt the deep seawater method in sea urchin aquaculture.

The use of salty groundwater for aquaculture, with a salinity comparable to seawater, is a recent development in Japan (Ebata *et al.*, 2006; Imada *et al.*, 2006). It can be obtained from wells bored near to the shore and has a stable temperature year round. In west Hokkaido, the temperature of salty groundwater is about 10℃ even in summer, although that of surface seawater rises over 20℃. The advantage of this method is that it requires small initial investment costs. However, this water sometimes contains high levels of ammonium and manganese ions (Ebata *et al.*, 2006). Such groundwater requires treatment using aeration and specified microbes to remove the ions before use (Ebata *et al.*, 2006).

Thus, there are multiple methods for decreasing the temperature of rearing water in summer. The chosen method depends on the location and scale of the aquaculture farm.

Acknowledgments

We are grateful to Ms. Rie Matsuda and Ms. Suzuko Tanaka at the Hokkaido National Fisheries

Research Institute for technical assistance throughout the study. This study was financially supported by the Fisheries Research Agency and the Hokkaido Research Organization.

References

Agatsuma Y., and Nishikiori T., 1991: Gonad development of the northern sea urchin, *Strongylocentrotus nudus*, that experimentally fed fishes. I. Gonad development. *Sci. Rep. Hokkaido Fish. Exp. Stn.*, **37**, 59–66 (in Japanese with English abstract).

Bay-Schmith E., and Pearse J. S., 1987: Effect of fixed day lengths on the photoperiodic regulation of gametogenesis in the sea urchin *Strongylocentrotus purpuratus*. *Int. J. Invertebr. Reprod. Dev.*, **11**, 287–294.

Böttger S. A., Devin M. G., and Walker C. W., 2006: Suspension of annual gametogenesis in North American green sea urchins (*Strongylocentrotus droebachiensis*) experiencing invariant photoperiod–Applications for land-based aquaculture. *Aquaculture*, **261**, 1422–1431.

Cochran R. C., and Engelmann F., 1975: Environmental regulation of the annual reproductive season of *Strongylocentrotus purpuratus* (Stimpson). *Biol. Bull.*, **148**, 393–401.

Dumont C., Pearce C. M., Stazicker C., An Y. X., and Keddy L., 2006: Can photoperiod manipulation affect gonad development of a boreo-arctic echinoid (*Strongylocentrotus droebachiensis*) following exposure in the wild after the autumnal equinox? *Mar. Biol.*, **149**, 365–378.

Ebata H., Sato Y., Fukue M., Shimada S., and Enokida K., 2006: Applicability of salty groundwater to aquaculture on land and its setback to be enhanced. *Bull. Soc. Sea Water Sci. Jpn.*, **60**, 110–118 (in Japanese with English abstract).

Fuji A., 1960a: Studies on the biology of the sea urchin I: Superficial and histological gonadal changes in gametogenic process of two sea urchins, *Strongylocentrotus nudus* and *S. intermedius*. *Bull. Fac. Fish. Hokkaido Univ.*, **11**, 1–14.

Fuji A., 1960b: Studies on the biology of the sea urchin I. Reproductive cycle of two sea urchins, *Strongylocentrotus nudus* and *S. intermedius*, in Southern Hokkaido. *Bull. Fac. Fish. Hokkaido Univ.*, **11**, 49–57

Fujita D., 2010: Current status and problems of isoyake in Japan. *Bull. Fish. Res. Agen.*, **32**, 33–42.

Fujita D., Kuwahara H., Watanuki A., Aota T., and Yokoyama J., 2008: Kokunai no uniyake no genjou. in "Recovery from urchin barrens – ecology, fishery, and utilization of sea urchins –" (ed. by Fujita D., Machiguchi Y., and Kuwahara H.), Seizando-Shoten, Tokyo, Japan, pp. 14–21 (in Japanese).

Garrido C. L., and Barber B. J., 2001: Effects of temperature and food ration on gonad growth and oogenesis of the green sea urchin, *Strongylocentrotus droebachiensis*. *Mar. Biol.*, **138**, 447–456.

Imada O., Maeda H., and Tanaka Y., 2006: Well seawater obtained from underground limestone layer in Okinawa Islands, its drawing techniques, physical, chemical and biological characteristics and performance of shellfish (abalone) cultivation. *Bull. Soc. Sea Water Sci. Jpn.*, **60**, 119–124 (in Japanese with English abstract).

Ito S., Shibayama M., Kobayakawa A., and Tani Y., 1989: Promotion of maturation and spawning of sea urchin *Hemicentrotus pulcherrimus* by regulating water temperature. *Nippon Suisan Gakkaishi*, **55**, 757–763 (in Japanese with English abstract).

Kato K. H., and Ishikawa M., 1982: Flagellum formation and centriolar behavior during spermatogenesis of the sea urchin, *Hemicentrotus pulcherrimus*. *Acta Embryol. Morphol. Exp. New Ser.*, **3**, 49–66.

Kayaba T., Tsuji K., Hoshikawa H., Kikuchi Y., Kawabata K., Otaki I., and Watanabe T., 2012: Effect of low temperature rearing, using deep-sea water, on gonadal maturation of the short-spined sea urchin, *Strongylocentrotus intermedius*, in Rausu, Hokkaido. *Fish. Sci.*, **78**, 1263–1272.

Kelly M. S., 2001: Environmental parameters controlling gametogenesis in the echinoid *Psammechinus miliaris*. *J. Exp. Mar. Biol. Ecol.*,

266, 67–80.

Kirchhoff N. T., Eddy S., and Brown N. P., 2010: Out-of-season gamete production in *Strongylocentrotus droebachiensis*: Photoperiod and temperature manipulation. *Aquaculture*, **303**, 77–85.

Marsh A. G., Powell M. L., and Watts S. A., 2013: Biochemical and energy requirements of gonad development. in "Sea urchins: biology and ecology, 3rd edn." (ed. by Lawrence J. M.), Elsevier, Amsterdam, Netherlands, pp. 45–57.

Masaki K., and Kawahara I., 1995: Promotion of gonadal maturation by regulating water temperature in the sea urchin *Pseudocentrotus depressus* I. *Bulletin of Saga Prefectural Sea Farming Center*, **4**, 93–100 (in Japanese).

Masuda R. J. C., and Dan S., 1977: Studies on the annual reproductive cycle of the sea urchin and the acid phosphatase activity of relict ova. *Biol. Bull.*, **153**, 577–590.

McCarron E., Burnell G., Kerry J., and Mouzakitis G., 2010: An experimental assessment on the effects of photoperiod treatments on the somatic and gonadal growth of the juvenile European purple sea urchin *Paracentrotus lividus*. *Aquacul. Res.*, **41**, 1072–1081.

McClintock J. B., and Watts S. A., 1990: The effects of photoperiod on gametogenesis in the tropical sea urchin *Eucidaris tribuloides* (Lamarck) (Echinodermata: Echinoidea), *J. Exp. Mar. Biol. Ecol.*, **139**, 175–184.

Murata Y., 2009: Aji no kagaku. in "Unigaku" (ed. by Motokawa T.), Tokai University Press, Kanagawa, Japan, pp. 205–220 (in Japanese).

Murata Y., Yokoyama M., Unuma T., Sata N. U., Kuwahara R., and Kaneniwa M., 2002: Seasonal changes of bitterness and pulcherrimine content in gonads of green sea urchin *Hemicentrotus pulcherrimus* at Iwaki in Fukushima Prefecture. *Fish. Sci.*, **68**, 184–189.

Nakasone T., and Akeda S., 2000: The application of deep sea water in Japan. *UJNR Technical Report*, **28**, 69–75.

Noguchi H., Kawahara I., Goto M., and Masaki K., 1995: Promotion of gonadal maturation by regulating water temperature in the sea urchin *Pseudocentrotus depressus* II. *Bulletin of the Saga Prefectural Sea Farming Center*, **4**, 101–107 (in Japanese).

Pearse J. S., 2006: Ecological role of purple sea urchins. *Science*, **314**, 940–941.

Pearse J. S., Pearse V. B., and Davis K. K., 1986: Photoperiodic regulation of gametogenesis and growth in the sea urchin, *Strongylocentrotus purpuratus*. *J. Exp. Zool.*, **237**, 107–118.

Reunov A. A., Yurchenko O. V., Kalachev A. V., and Au D. W. T., 2004: An ultrastructural study of phagocytosis and shrinkage in nutritive phagocytes of the sea urchin *Anthocidaris crassispina*. *Cell Tissue Res.*, **318**, 419–428.

Sakai Y., 2015: Hatchery technology (production of seed). in "Aquaculture of Echinoderms" (ed. by Brown N. P., and Eddy S. D.), John Wiley and Sons, New York, NY, (in press).

Sakairi K., Yamamoto M., Ohtsu K., and Yoshida M., 1989: Environmental control of gonadal maturation in laboratory-reared sea urchins, *Anthocidaris crassispina* and *Hemicentrotus pulcherrimus*. *Zool. Sci.*, **6**, 721–730.

Shpigel M., McBride S. C., Marciano S., and Lupatsch I., 2004: The effect of photoperiod and temperature on the reproduction of European sea urchin *Paracentrotus lividus*. *Aquaculture*, **232**, 343–355.

Spirlet C., Grosjean P., and Jangoux M., 2000: Optimization of gonad growth by manipulation of temperature and photoperiod in cultivated sea urchins, *Paracentrotus lividus* (Lamarck) (Echinodermata), *Aquaculture*, **185**, 85–99.

Tominaga A., and Takashima Y., 1987: Ultracytochemical study on the behavior of the nurse cell giant granules in the sea urchin ovary. *Acta Histochem. Cytochem.*, **20**, 569–579.

Unuma T., 2002: Gonadal growth and its relationship to aquaculture in sea urchins. in "The sea urchin: From basic biology to aquaculture" (ed. by Yokota Y., Matranga V., and Smolenicka Z.), Swets&Zeitlinger, Lisse, Netherlands, pp. 115–127.

Unuma T., 2009: Uni wo oishiku taberu. in "Unigaku" (ed. by Motokawa T.), Tokai University Press, Kanagawa, Japan, pp. 159–179 (in Japanese).

Unuma T., 2015: Sea urchin fisheries in Japan. in "Aquaculture of Echinoderms" (ed. by Brown N.

P., and Eddy S. D.), John Wiley and Sons, New York, NY, (in press).

Unuma T., and Kayaba T., 2015: Land-based and captive sea-based grow-out (cultivation of seed to market size). in "Aquaculture of Echinoderms" (ed. by Brown N. P., and Eddy S. D.), John Wiley and Sons, New York, NY, (in press).

Unuma T., and Walker C. W., 2009: Relationship between gametogenesis and food quality in sea urchin gonads. in "Aquaculture technologies for invertebrates: Proceedings of the thirty-sixth U.S.-Japan aquaculture panel symposium" (ed. by Stickney R., Iwamoto R., and Rust M.), U.S. Dept. Commerce, NOAA Tech. Memo pp. 45-54.

Unuma T., Sawaguchi S., Yamano K., and Ohta H., 2011: Accumulation of the major yolk protein and zinc in the agametogenic sea urchin gonad. *Biol. Bull.*, **221**, 227-237.

Unuma T., Yamamoto T., Akiyama T., Shiraishi M., and Ohta H., 2003: Quantitative changes in yolk protein and other components in the ovary and testis of the sea urchin *Pseudocentrotus depressus*. *J. Exp. Biol.*, **206**, 365-372.

Walker C. W., 1982: Nutrition of gametes. in "Echinoderm nutrition" (ed. by Jangoux M., and Lawrence J. M.), Balkema, Rotterdam, Netherlands. pp. 449-468.

Walker C. W., and Lesser M. P., 1998: Manipulation of food and photoperiod promotes out-of-season gametogenesis in the green sea urchin *Strongylocentrotus droebachiensis*: Implications for aquaculture. *Mar. Biol.*, **132**, 663-676.

Walker C. W., Harrington L. M., Lesser M. P., and Fagerberg W. R., 2005: Nutritive phagocyte incubation chambers provide a structural and nutritive microenvironment for germ cells of *Strongylocentrotus droebachiensis*, the green sea urchin. *Biol. Bull.*, **209**, 31-48.

Walker C. W., Lesser M. P., and Unuma T., 2013: Sea urchin gametogenesis - structural, functional and molecular/genomic biology. in "Sea urchins: biology and ecology, 3rd edn." (ed. by Lawrence, J. M.), Elsevier, Amsterdam, Netherlands, pp. 25-38.

Ward R. D., and Nishioka D., 1993: Seasonal changes in testicular structure and localization of a sperm surface glycoprotein during spermatogenesis in sea urchins. *J. Histochem. Cytochem.*, **41**, 423-431.

Yamamoto M., Ishine M., and Yoshida M., 1988: Gonadal maturation independent of photic conditions in laboratory-reared sea urchins, *Pseudocentrotus depressus* and *Hemicentrotus pulcherrimus*. *Zool. Sci.*, **5**, 979-988.

水研センター研報，第40号，155－165，平成27年
Bull. Fish. Res. Agen. No. 40, 155－165, 2015

Maturation Control of the Short-Spined Sea Urchin, *Strongylocentrotus intermedius*, by Low Temperature Rearing Using Deep-Sea Water, with the Aim of Extending the Market Season

Takaaki KAYABA[*1], Kohji TSUJI[*2], Hiroshi HOSHIKAWA[*3], Yaoki KIKUCHI[*4], Kazuhiro KAWABATA[*4], Isao OTAKI[*4], Toru WATANABE[*5]

Abstract: The short-spined sea urchin *Strongylocentrotus intermedius*, is a valuable commercial species and one of the most popular and expensive seafoods in Japan. Serving local short-spined sea urchin to visitors during the summer tourist season in Rausu, located in a world natural heritage site "Shiretoko," has long been desired. However, it has not been feasible during the sea urchin spawning season (July to September), resulting in quality degradation in gonads, the edible part of sea urchins, due to maturation. Therefore, the Hokkaido Research Organization and the Rausu Fishery Cooperative Associations cooperatively investigated the possibility of suppressing gonadal maturation and maintaining high quality sea urchin gonads by low temperature rearing using deep-sea water. Unripe sea urchins captured before the spawning season were reared under two temperature conditions from June to September. In groups reared at ambient temperatures (2–18℃), gametogenesis in both sexes progressed rapidly with increased temperature, and almost all sea urchins reached full maturity by late July. Whereas in groups reared at low temperatures (2–5℃), gametogenesis progressed slowly and over 60% of the sea urchins had not reached maturity even by early September. This result suggests that the progress of gametogenesis in the short-spined sea urchin is effectively suppressed by rearing under low temperature conditions. Additionally, we also examined the effects of feeding on gonadal development in sea urchins reared under low temperature conditions, and revealed that feeding with live *Saccharina diabolica*, which were cultured as food in Rausu, could increase the gonadal volume efficiently to commercially preferable size, while suppressing the progress of gametogenesis. Moreover, the results of chemical analysis and tasting test proved that the quality of gonads were very excellent in sea urchin fed with live *S. diabolica*. Consequently, we demonstrate that low temperature rearing, supplemented with feeding live *S. diabolica*, is effective in suppressing gametogenesis to allow for the harvesting of high quality sea urchins during the summer tourist season. At present, this aquaculture method is being put to practical use by Rausu Fishery Cooperative.

Key words: short-spined sea urchin *Strongylocentrotus intermedius*, gametogenesis, deep-sea water, low temperature rearing, sea urchin aquaculture

The short-spined sea urchin *Strongylocentrotus intermedius*, which is distributed on the northern coast of Japan, is a valuable commercial species and is one of the most popular and expensive seafoods in the country; more than 2000 tons of the sea urchin are caught annually in Hokkaido, the main harvest region for this species. *S. intermedius* has bright orange gonads with a rich taste, and the gonads

2015年1月30日受理（Received on January 30, 2015）
[*1] Kushiro Fisheries Research Institute, Hokkaido Research Organization, Kushiro, Hokkaido 085-0024, Japan
[*2] Abashiri Fisheries Research Institute, Hokkaido Research Organization, Monbetsu, Hokkaido 094-0011, Japan
[*3] Central Fisheries Research Institute, Hokkaido Research Organization, Yoichi, Hokkaido 046-8555, Japan
[*4] Department of Mariculture, Rausu Fishery Cooperative, Hokkaido Research Organization, Rausu, Hokkaido 086-1893, Japan
[*5] Hachinohe Scientific Museum MARIENT, Hachinohe, Aomori, 031-0841, Japan
Corresponding author: Takaaki KAYABA E-mail: kayaba-takaaki@hro.or.jp

are preferred especially as an attractive raw food material, such as "Sushi" or "Sashimi (Uni-don)". *S. intermedius* has also become one of the important tourist resources of Hokkaido, because many tourists visit Hokkaido in anticipation of the tastes of fresh sea urchin every year.

Since the edible part of the sea urchin is usually restricted to the gonads, its quality as food varies dramatically with gonadal growth and maturation. In general, the superior quality of gonads, defined by attractive taste, color, and shape, is only present during the term of the unripe phase when gametogenesis is limited. The mature gonad has an unpleasant taste due to the gamete contents and a melting appearance caused by the flow of gametes (Unuma, 2002). Therefore, the best season for harvesting *S. intermedius* is very short—less than three months per a fishery region —in spite of an annual market demand. There would be considerable commercial value in developing an aquaculture technique to control the timing of gonadal maturity so as to ensure a supply of high quality sea urchins over a longer period.

Rausu town, located on the Shiretoko Peninsula in east Hokkaido, is a prominent production region of *S. intermedius* in Japan (Fig. 1). After Shiretoko Peninsula was registered as a world natural heritage site in 2005, increasing numbers of tourists have visited Rausu, especially during the summer, from July to September. In Rausu, there is a huge demand for serving *S. intermedius* to visitors during the summer tourist season. However, it has been exceedingly difficult to supply high quality sea urchins during the summer, because the spawning season of sea urchins in Rausu is from July to September and the quality of gonads degrades on maturation. Moreover, in Rausu, as a resource conservation measure, harvesting sea urchins from July to September is legally prohibited by local government fishing regulations. Therefore, it is necessary to develop a method for culturing *S. intermedius* caught before the closed season, so as to maintain their food quality by suppressing maturation.

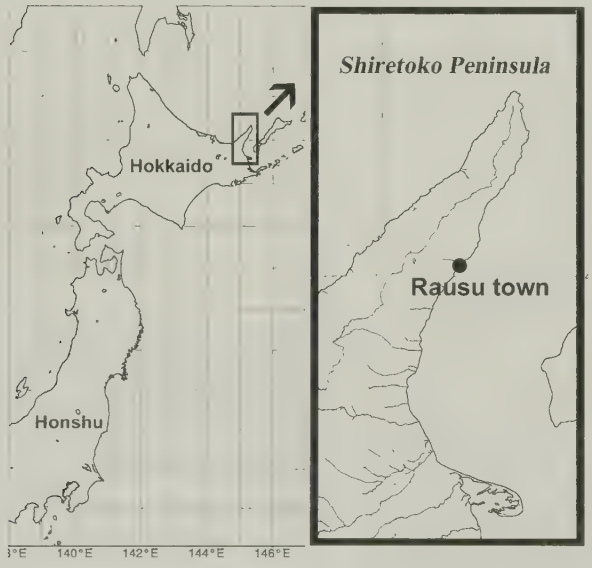


Fig. 1. Location of Rausu town on the Shiretoko Peninsula in east Hokkaido, Japan.

Annual reproductive cycles of sea urchins are regulated by environmental factors, such as water temperature (Yamamoto *et al.*, 1988; Sakairi *et al.*, 1989), photoperiod (Pearse *et al.*, 1986; McClintock and Watts, 1990; Walker and Lesser, 1998; Böttger *et al.*, 2006), and lunar period (Horii, 1997; Coppard and Campbell, 2005). Water temperature in particular has been shown to have a strong effect on the progress of gametogenesis in sea urchins. In some Japanese urchin hatcheries, temperature control is used to induce broodstocks of *Pseudocentrotus depressus, Hemicentrotus pulcherrimus*, and *S. intermedius* to mature out of the normal spawning season (Ito *et al.*, 1989; Masaki and Kawahara, 1995; Noguchi *et al.*, 1995). The gonadal development of wild *S. intermedius* tends to be correlated with a rise in water temperature from spring to summer (Tomita *et al.*, 1984). Accordingly, it may be possible to suppress gametogenesis by rearing sea urchins under low temperature conditions during that period.

Generally, using electric cooling devices in aquaculture is limited because of its high costs. Fortunately, a facility for collecting deep-sea water is available in Rausu. The deep-sea water is defined as seawater that is pumped from a depth of greater than 200 m, and has certain advantageous properties: stable low temperature, low in suspended particles and bacteria, and rich in nutrient salts (Nakasone and Akeda, 2000). More than ten facilities capable of drawing deep seawater have been constructed in Japan, where it is used for aquaculture, handling

of captured fishes, the food industry, medical treatment, and agriculture (Takahashi, 2006). In Rausu, deep seawater is drawn from a depth of 350 m and has been used mainly as a raw material for refining mineral water and as rearing water to temporarily hold captured salmon. Additionally, there is a sufficient supply of deep-sea water, such that it has been collected constantly and used for aquaculture in Rausu. Fig. 2 indicates variations in water temperature between the deep-sea water and ambient sea water drawn from the surface. The water temperature of deep seawater is stable at lower degrees from March to September compared with the ambient seawater, ranging from 2 to 5℃. Therefore, taking advantage of the available facility, the Hokkaido Research Organization and the Rausu Fishery Cooperative Associations cooperatively investigated the effects of rearing under low temperature conditions on the gametogenesis of *S. intermedius*, in an effort to develop reliable techniques for maintaining gonads in good quality during the summer tourist season in Rausu, as a model (Kayaba *et al.*, 2012). In this proceeding, we describe new aquaculture technique of sea urchin and its possibility in the future, adding further data to the results which have been reported in Kayaba *et al.* (2012).

The effects of rearing under low temperature conditions on gametogenesis

To determine the effect of water temperature on suppressing gametogenesis, rearing experiments were conducted under two types of water temperature conditions from 2008 to 2010 (Kayaba *et al.*, 2012). Unripe sea urchins (weighing 54.0–81.3 g), were collected from the coastal fishery ground at Rausu in March – May. They were divided into four experimental cages (100 × 100 × 80 mm) at densities of 150 individuals per cage, and transferred to the experimental environment on June 1 of each year. Two cages of sea urchins were maintained with running ambient sea water (2.8 – 19.6℃) until September 1 (ambient temperature group), while the other two cages were maintained with running deep-sea water (2.5 – 4.9℃) over the same period (deep seawater temperature group). In addition, for determining the effects of feeding on gametogenesis and gonad size, individuals in one cage of both temperature groups were reared without feeding (non-feeding cage), while individuals in the other cages were given a surplus of commercial dry wakame (*Undaria sp.*, obtained from the Riken Vitamin Company, Tokyo) every day (feeding cage). Both groups of sea urchins were reared in the dark throughout the experimental period, except

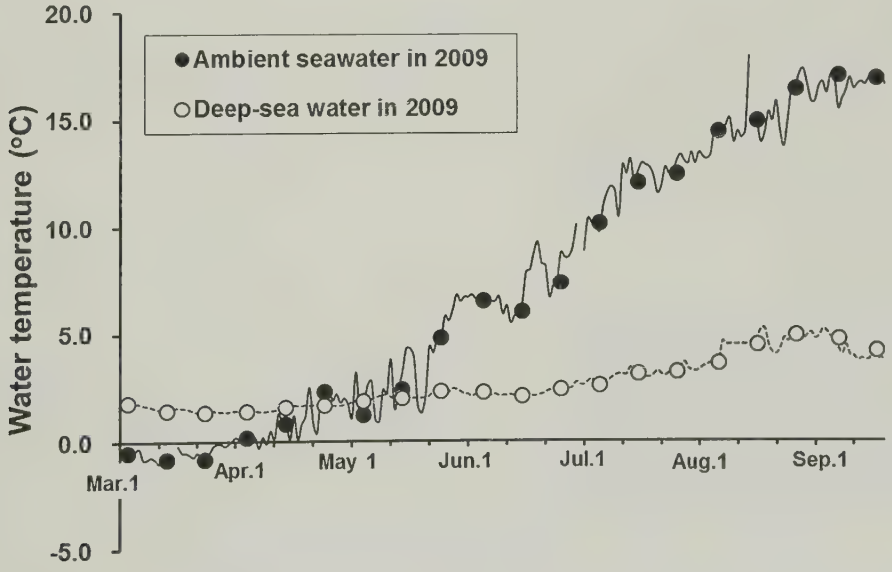


Fig. 2. Variations in temperature of the ambient sea water drawn from the surface of the Rausu coast and that of the deep-sea water drawn from a depth of 350 m off the coast of Rausu, in 2009.

for during feeding and cleaning of excreta. Twenty individuals were sampled from each group monthly for histological observation of gonads. Maturational status of ovaries and testes was classified into five stages according to Fuji (1960); stage 1 (before gametogenesis), stage 2 (early gametogenesis), stage 3 (mid-gametogenesis), stage 4 (fully mature) and stage 5 (spent). Gonads at stages 1 to 3 are commercially favorable. On the other hand, the fully mature gonads (stage 4) were not suitable for consumption because of the unpleasant taste caused by the gamete contents and a melting appearance caused by the flow of gametes via breakage of the gonoduct (Unuma, 2002). The spent gonads (stage 5) were also unsuitable for food, because they were shrunken.

Table 1 shows the frequency of maturation stage of female and male sea urchins during the experiment in 2009. In the ambient temperature group, gametogenesis progressed rapidly with increased temperature and almost all sea urchins reached full maturity (stage 4) by 27 July, about 2 months after the initiation of the experiment. In contrast, gametogenesis in both ovaries and testes progressed slowly in the deep seawater temperature group, and about 60 % of the sea urchins were maintained at an unripe stage (stages 1–3) until 31 August. In spite of distinct differences in maturity among two rearing temperature conditions, significant differences in the distribution of maturation stage could not be found between sea urchins in feeding cages and non-feeding cages in both sexes. These results strongly suggest that the progress of gametogenesis in *S. intermedius* is closely related to environmental temperature, and is effectively suppressed by rearing sea urchins under low temperature conditions. Following repeated tests over three years, it was proven that rearing at deep seawater temperatures could extend the harvest season two months or longer beyond that of the wild population (Kayaba *et al.*, 2012). Therefore, the techniques used in our study, for maintaining unripe sea urchins under low temperature conditions, are likely candidates for an efficient method for inhibiting gonadal maturation and serving sea urchins with good quality during summer tourist season.

Table 1. The frequency of maturation stage of female and male sea urchins reared at ambient temperature or deep seawater temperature during the experiment performed in 2009 (based on data from Kayaba *et al.*, 2012).

Sex	Feeding condition	Date	Ambient temperature group					Deep seawater temperature group				
			Favorable maturity			Unfavorable maturity		Favorable maturity			Unfavorable maturity	
			Stage 1	Stage 2	Stage 3	Stage 4	Stage 5	Stage 1	Stage 2	Stage 3	Stage 4	Stage 5
Female	Feeding	27-May	0.00	0.71	0.21	0.07	0.00	0.00	0.71	0.21	0.07	0.00
		1-Jul	0.00	0.50	0.50	0.00	0.00	0.00	0.67	0.33	0.00	0.00
		27-Jul	0.00	0.00	0.22	0.78	0.00	0.00	0.29	0.43	0.14	0.14
		31-Aug	0.17	0.00	0.00	0.33	0.50	0.10	0.00	0.70	0.20	0.00
	Non -feeding	27-May	0.00	0.71	0.21	0.07	0.00	0.00	0.71	0.21	0.07	0.00
		1-Jul	0.00	0.20	0.60	0.20	0.00	0.00	0.33	0.50	0.17	0.00
		27-Jul	0.00	0.00	0.30	0.70	0.00	0.00	0.27	0.45	0.27	0.00
		31-Aug	0.00	0.00	0.00	0.33	0.67	0.00	0.20	0.40	0.20	0.20
Male	Feeding	27-May	0.00	0.38	0.38	0.25	0.00	0.00	0.38	0.38	0.25	0.00
		1-Jul	0.00	0.00	0.38	0.63	0.00	0.00	0.43	0.57	0.00	0.00
		27-Jul	0.00	0.00	0.00	1.00	0.00	0.00	0.08	0.77	0.15	0.00
		31-Aug	0.00	0.00	0.00	0.75	0.25	0.00	0.00	0.60	0.40	0.00
	Non -feeding	27-May	0.00	0.38	0.38	0.25	0.00	0.00	0.38	0.38	0.25	0.00
		1-Jul	0.00	0.00	0.50	0.40	0.10	0.00	0.00	0.80	0.20	0.00
		27-Jul	0.00	0.00	0.00	0.71	0.29	0.00	0.00	0.67	0.33	0.00
		31-Aug	0.00	0.00	0.00	0.50	0.50	0.00	0.00	0.53	0.33	0.13

Improvement in quality of short-spined sea urchins reared under low temperature conditions

In general, the sea urchins with larger-sized unripe gonads are commercially preferred for consumption. Therefore, for aquaculture of sea urchins, it is also important to determine the rearing condition by which gonads grow faster to a commercial size. For echinoderms, it has been established that nutritive condition is closely related to gonadal development. Nutrients derived from ingested food are stored in nutritive phagocytes inside the ovary and testis, and are utilized for production or growth of germ cells (Unuma, 2002; Walker *et al.*, 2006). In the rearing experiment mentioned above, obvious increases in gonad volume were observed only in the sea urchins reared with feeding: the gonad indexes (GIs) of sea urchins reared with feeding were significantly higher than those without feeding (Fig. 3). This result indicates obviously that rearing with feeding under low temperature conditions is the best way to accelerate the accumulation of nutrients without proceeding gametogenesis in gonads of *S. intermedius*.

Accordingly, as a next step, we examined the dietary effects of two available diets in Rausu, commercial dry wakame and live brown macroalgae, *Saccharine diabolica*; the former had the advantage of low price and ready availability, while the later was the primary food plant for wild *S. intermedius* and was flourishingly cultured as food in Rausu (Kayaba *et al.*, 2012). *S. intermedius* were reared with four feeding regimes at deep-sea water temperatures from June 1 to September 1 in 2009. In the first group, the sea urchins were reared without feeding during the experimental period as a control. In the second and third groups, the sea urchins were fed a surplus of dry wakame from June 1 to September 1 (feeding for 3 months) and from August 1 to September 1 (feeding for 1 month), respectively. In the fourth group, the sea urchins were fed an unlimited amount of live brown macroalgae, *S. diabolica* from August 1 to September 1 (feeding for 1 month). In comparing GIs from each treatment, when sea urchins were fed live *S. diabolica*, the growth rate of gonads was three times as fast as that when they were fed dry wakame (Table 2). The daily food consumption for each diet was almost the same, suggesting that for *S. intermedius*, *S. diabolica* may be easier to assimilate. Several feeding experiments have shown that the *Laminariales* are the most nutritionally valuable algae among several algae distributed along the coast of Hokkaido for promoting gonadal growth and

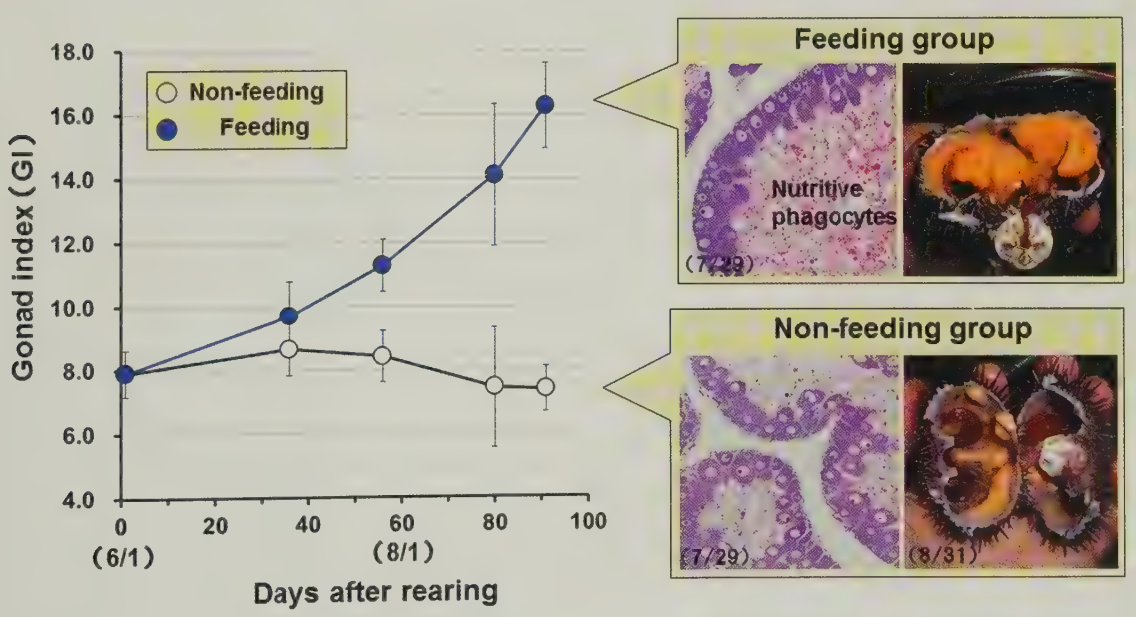


Fig. 3. Changes in the gonad index (GI) and histological structures of *S. intermedius* reared at deep seawater temperature under different feeding regimes during the experiment performed in 2009. Values represent the mean ± standard deviation (based on data from Kayaba *et al.*, 2012).

Table 2. Changes in the gonad index (GI) of *S. intermedius* reared with four feeding regimes at low temperatures during the experiment performed in 2009 (based on data from Kayaba *et al.*, 2012).

Values represent the mean ± standard deviation. Mean values with different letters are significantly different at the 0.05 level within each sampling day. (GIs were arcsine-transformed before analysis and reported as back-transformed values. When a treatment effect was significant, Tukey's multiple range comparison test was applied to identify differences among experimental groups.)

Sampling date	*Test group*	1	2	3	4
	Diet	Non-feeding	Dry wakame	Dry wakame	Live *S. diabolica*
	Feeding term	—	3 months	1 month	1 month
	Feeding period	—	1 Jun — 1 Sep	1 Aug — 1 Sep	1 Aug — 1 Sep
27-May (Initial)		7.9 ± 0.7	7.9 ± 0.7	7.9 ± 0.7	7.9 ± 0.7
1-Jul		8.5 ± 0.8	9.7 ± 1.1	8.7 ± 0.8	8.7 ± 0.8
27-Jul		8.4 ± 0.8 **b**	11.3 ± 0.8 **a**	8.2 ± 0.8 **b**	8.4 ± 0.8 **b**
20-Aug		7.4 ± 1.9 **c**	14.1 ± 2.2 **a**	10.7 ± 0.9 **b**	9.9 ± 0.5 **b**
31-Aug		7.4 ± 0.7 **c**	16.3 ± 1.4 **a**	11.0 ± 1.4 **b**	15.6 ± 1.6 **a**

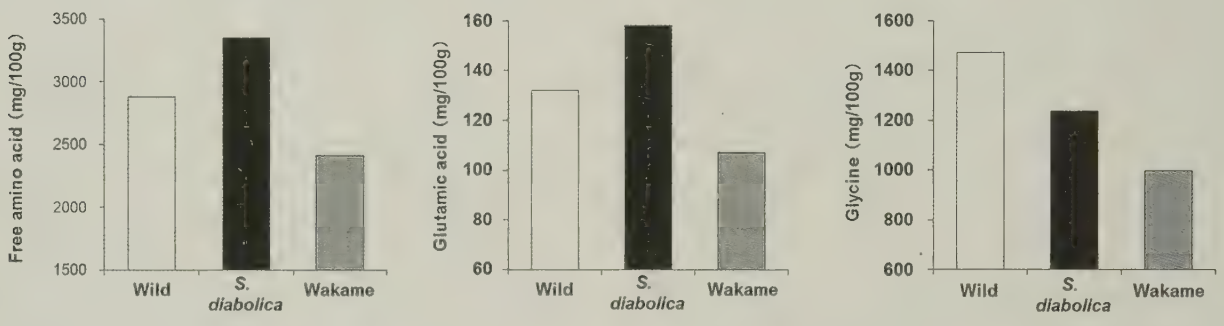


Fig. 4. Effect of diet on the free amino acid content of gonads in *S. intermedius* reared under different feeding conditions. Wild: wild sea urchins captured from the fishing ground in Rausu, *S. diabolica*: sea urchins reared and fed live *S. diabolica* for one month, Wakame: sea urchins reared and fed commercial dry wakame for one month

improving the taste of gonads in the northern sea urchin *Strongylocentrotus nudus*, a sub-arctic species like the *S. intermedius* (Agatsuma *et al.*, 1993; Nabata *et al.*, 1999). Accordingly, as demonstrated in our experiments with *S. intermedius*, it may be possible, by feeding with live *S. diabolica*, to promote gonadal growth efficiently even under low temperature conditions, while suppressing the progress of gametogenesis.

Moreover, we also investigated the characteristic of taste in sea urchins reared with feeding live *S. diabolica* by chemical analysis (free amino acids) or tasting test directed to tourists in Rausu. As a result, the gonads of sea urchins fed live *S. diabolica* contained in rich amount of glutamic acid flavoring "Umami taste" and glycine flavoring "Sweetness", comparing with those fed dry wakame (Fig. 4). Furthermore, tasting test proved that the taste of gonads in sea urchin fed with live *S. diabolica* were very preferable to customers (Fig. 5). These results clearly indicate that live *S. diabolica* is an essential diet for rearing *S. intermedius* to not only enlarge their gonad volume, but also to improve the quality of gonads. Generally, it has been suggested that the largest limiting factor for the success of sea urchin aquaculture on a commercial scale is the difficulty of obtaining sufficient macroalgae for feeding. Fortunately, in the cultivation of *S. diabolica* in Rausu town, a large amount of surplus algae is produced every early summer by thinning to accelerate its growth. It will be capable of producing commercially viable sea urchins sustainably and at a

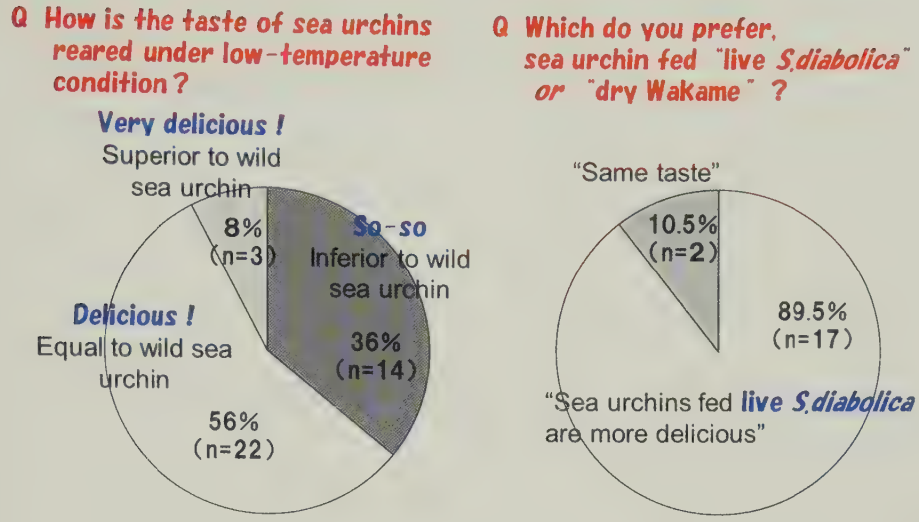

Q How is the taste of sea urchins reared under low-temperature condition?
Very delicious !
Superior to wild sea urchin
8% (n=3)
So-so
Inferior to wild sea urchin
36% (n=14)
Delicious !
Equal to wild sea urchin
56% (n=22)
Q Which do you prefer, sea urchin fed "live S.diabolica" or "dry Wakame" ?
"Same taste"
10.5% (n=2)
89.5% (n=17)
"Sea urchins fed live S.diabolica are more delicious"

Fig. 5. A questionnaire to ask the tourists visited to Rausu in August 2010, for investigation of the practicability of low-temperature rearing system in *S. intermedius*.

Collecting unripe sea urchins
Extending in market season
①Low-temperature rearing by deep-sea water
• Suppression in gametogenesis
Low cost
Using the unutilized algae as diet
②③ Feeding live S.diabolica
• Increase in volume of gonad
• Improving taste
Preferable quality
New, attractive food item "Shiletoko sea urchin"!!
① Good appearance !
② Good volume !
③ Good taste !

Fig. 6. The best practical use of *S. intermedius* in Rausu, with the aim of extending market season.

lower cost by using this source of unutilized algae as a diet.

In conclusion and future prospects

Our present study was initiated to suppress the gametogenesis of unripe *S. intermedius* captured before the spawning season by low temperature rearing using deep-sea water. We succeeded in extending the harvest season of sea urchins reared with deep-sea water two months longer than that of the wild population, and proved that serving them during the summer tourist season was certainly possible. The best practical use of *S. intermedius* in Rausu would include the following steps: 1) a large number of *S. intermedius* are caught from the wild toward the end of the fishing season (late June), 2) sea urchins are reared in tanks supplied

with deep-sea water to suppress their maturation until harvest, and 3) sea urchins are fed mainly on live *S. diabolica* for one month before harvest to improve gonadal growth (Fig. 6). During August, the peak of the summer tourist market in Rausu, the probability of harvesting sea urchins suitable for consumption is approximately 70% per total reared sea urchins. Further detailed studies concerning the rearing conditions of sea urchins would increase that probability in the future.

At present, this aquaculture method is being put to practical use by Rausu Fisheries Cooperative. There is a plan of serving sea urchins at seafood restaurants, hotels, and summer festivals that are visited by many tourists, and they are expected to be a new, attractive food item which will help to promote the popularity of "Shiretoko" as a tourist destination. Should this trial be successful, it will provide a good case for presenting the potential of sea urchin aquaculture to expand not only local fishery production, but also tourism in the local area.

Recently, the extent of barren grounds has increased in coastal areas around Hokkaido. An excessive grazing of seaweeds by wild sea urchins is suspected as one of cause of this phenomenon (urchin-dominated barren ground, called "uniyake" in Japanese) (Fujita, 2008). To recover the luxuriant growth of seaweed, large numbers of sea urchins have been removed from these barren grounds and destroyed, because they have poor gonads due to a shortage of food and have no commercial value (Agatsuma *et al.*, 1997; Kuwahara *et al.*, 2010). The present culture technique would make it possible to improve the quality of surplus wild sea urchins removed from the barren ground so they are preferred by customers. The technique may also become a useful method to create a balance between the biomass of seaweed and the population of sea urchins in coastal fishing grounds. In the future, it is expected that this culture method will be a valuable tool for conserving the coastal fishing grounds in Hokkaido.

Acknowledgment

This study was conducted as part of a research project, "Research and Development Projects for Application in the New Policy of Agriculture, Forestry and Fisheries," funded by the Ministry of Agriculture, Forestry and Fisheries, Japan.

Reference

Agatsuma Y., Nakata A., and Matsuyama K., 1993: Feeding and assimilation of the sea urchin, *Strongylocentrotus nudus for Laminaria religiosa*. *Sci. Rep. Hokkaido Fish. Exp. Stn.*, **40**, 21–29. (in Japanese with English abstract)

Agatsuma Y., Matsuyama K., Nakata A., Kawai T., and Nishikawa N., 1997: Marine algal succession on coralline flats after removal of sea urchins in Suttsu Bay on the Japan Sea coast of Hokkaido, Japan. *Nippon Suisan Gakkaishi*, **63**, 672–680. (in Japanese with English abstract)

Böttger S. A., Devin M. G., and Walker C. W., 2006: Suspension of annual gametogenesis in green sea urchins experiencing invariant photoperiod – Applications for land-based aquaculture. *Aquaculture*, **261**, 1422–1431.

Coppard S. E., and Campbell A. C., 2005: Lunar periodicities of diadematid echinoids breeding in Fiji. *Coral Reefs*, **24**, 324–332.

Fuji A., 1960: Studies on the biology of the sea urchin. I. Superficial and histological gonadal changes in gametogenic process of two sea urchins, *Strongylocentrotus nudus and S. intermedius*. *Bull. Fac. Fish. Hokkaido Univ.*, **11**, 1–14.

Fujita D., Kuwahara H., Watanuki A., Aota T., and Yokoyama J., 2008: Kokunai no uniyake no genjou, in "Recovery from urchin barrens – ecology, fishery, and utilization of sea urchins" (ed. by Fujita D., Machiguchi Y., and Kuwahara H.), Seizando-Shoten, Tokyo, pp. 14–21. (in Japanese)

Horii T., 1997: The annual reproductive cycle and lunar spawning rhythms of the purple sea urchin *Anthocidaris crassispina* (in Japanese with English abstract). *Nippon Suisan Gakkaishi*, **63**, 17–22.

Ito S., Shibayama M., Kobayakawa M. A., and Tani Y., 1989: Promotion of maturation and spawning of sea urchin *Hemicentrotus pulcherrimus* by regulating water temperature (in Japanese with

English abstract). *Nippon Suisan Gakkaishi*, **55**, 757-763.

Kayaba T., Tsuji K., Hoshikawa H., Kikuchi Y., Kawabata K., Otaki I., and Watanabe T., 2012: Effect of low temperature rearing, using deep-sea water, on gonadal maturation of the short-spined sea urchin, *Strongylocentrotus intermedius*, in Rausu, Hokkaido. *Fish. Sci.*, **78**, 1263-1272.

Kuwahara H., Hashimoto O., Sato A., and Fujita D., 2010: Introduction of isoyake recovery guideline (Fisheries Agency, Japan). *Bull. Fish. Res. Agen.*, **32**, 51-60.

Masaki K., and Kawahara I., 1995: Promotion of gonadal maturation by regulating water temperature in the sea urchin *Pseudocentrotus depressus* - I (in Japanese). *Bull. Saga Pref. Sea Farming Center*, **4**, 93-100.

McClintock J. B., and Watts S. A., 1990: The effects of photoperiod on gametogenesis in the tropical sea urchin *Euchidaris tribuloides* (Lamarck) (Echinodermata: Echinoidea). *J. Exp. Mar. Biol. Ecol.*, **139**, 175-184.

Nabata S., Hoshikawa H., Sakai Y., Funaoka T., Oohori T., and Imamura T., 1999: Food value of several algae for growth of the sea urchin, *Strongylocentrotus nudus* (in Japanese with English abstract). *Sci. Rep. Hokkaido Fish. Exp. Stn.*, **54**, 33-40.

Nakasone T., and Akeda S., 2000: The application of deep sea water in Japan. UJNR *Technical Report*, **28**, 69-75.

Noguchi H., Kawahara I., Goto M., and Masaki K., 1995: Promotion of gonadal maturation by regulating water temperature in the sea urchin *Pseudocentrotus depressus* -II (in Japanese). *Bull. Saga Pref. Sea Farming Center*, **4**, 101-107.

Pearse J. S., Pearse V. B., and Davis K. K., 1986: Photoperiodic regulation of gametogenesis and growth in the sea urchin *Strongylocentroutus purpuratus*. *J. Exp. Zool.*, **237**, 107-118.

Sakairi K., Yamamoto M., Ohtsu K., and Yoshida M., 1989: Environmental control of gonadal maturation in laboratory-reared sea urchin, *Anthocidaris crassispina and Hemicentrotus pulcherrimus*. *Zool. Sci.*, **6**, 721-730.

Takahashi S., 2006: Deep seawater, in "Utilization of deep seawater resources" (ed. by Fujita D., and Takahashi S.,), Seizando-shoten, Tokyo, pp. 1-27.

Tomita K., Kishida M., Masusaki K., and Iisawa N., 1984: Seasonal changes in gonado of short-spined sea urchin *Strongylocentrotus intermedius* in the coast of East Hokkaido (in Japanese). *Monthly report of Hokkaido Fish Exp. Station*, **41**, 469-479.

Unuma T., 2002: Gonadal growth and its relationship to aquaculture in sea urchins, in "The sea urchin: from basic biology to aquaculture" (ed. by Yokota *et al.*), Swets & Zeitlinger, Lisse, pp. 115-127.

Yamamoto M., Ishine M., and Yoshida M., 1988: Gonadal maturation independent of photic conditions in laboratory-reared sea urchins, *Pseudocentrotus depressus* and *Hemicentrotus pulcherrimus*. *Zool. Sci.*, **5**, 979-988.

Walker C. W., and Lesser M. P., 1998: Manipulation of food and photoperiod promotes out-of season gametogenesis in the green sea urchin *Strongylocentrotus droebachiensis*: implications for aquaculture. *Mar. Biol.*, **132**, 663-676.

Walker C. W., Unuma T., and Lesser M. P., 2006: Gametogenesis and reproduction of sea urchins, in "Edible sea urchins: Biology and ecology" (Lawrence *et al.*), Elsevier, Amsterdam, pp. 11-33.

Annotated Bibliography

Unuma T., 2002: Gonadal growth and its relationship to aquaculture in sea urchins, in "The sea urchin: from basic biology to aquaculture" (ed. by Yokota *et al.*), Swets & Zeitlinger, Lisse, pp. 115-127.

The sea urchin gonad contains two main types of cells: nutritive phagocytes (NPs) and germ cells (GCs). NPs store nutrients necessary for gametogenesis, such as proteins, carbohydrates and lipids. The most abundant protein in NPs is a glycoprotein called the major yolk protein (MYP), originally identified as the most predominant component of yolk granules in the eggs. The proportion of NPs and GCs varies with the maturation of the gonads. Before gametogenesis, NPs fill the gonadal lumina and increase in size by accumulating nutrients to the developing GCs and

decrease in size. In fully mature gonads, a number of type ova or spematozoa fill the gonadal lumina, whereas, NPs lose their nutrients and shrink. The quality of the gonad as a food product usually deteriorates while GCs proliferate and develop. Promotion of NP growth and suppression of gametogenesis are prerequisites to achieving high profitability in sea urchin aquaculture.

Kayaba T., Tsuji K., Hoshikawa H., Kikuchi Y., Kawabata K., Otaki I., and Watanabe T., 2012: Effect of low temperature rearing, using deep-sea water, on gonadal maturation of the short-spined sea urchin, *Strongylocentrotus intermedius*, in Rausu, Hokkaido. *Fish. Sci.*, **78**, 1263–1272.

It has long been hoped that sea urchins could be served to visitors in seafood restaurants, hotels, and summer festivals in Rausu, located in a world natural heritage site "Shiretoko," during the summer tourist season. However, to date this has not been feasible because of the sea urchin spawning season (July to September), during which the quality of gonads, the edible part of sea urchins, decreases due to maturation. In this study, we examined the possibility of suppressing gonadal maturation and maintaining high-quality sea urchin gonads by low-temperature rearing using deep-sea water. Unripe sea urchins captured before the spawning season were reared under two temperature conditions from June to September. In those groups reared at ambient temperatures (2.8–19.6℃), gametogenesis in both sexes progressed rapidly with increased temperature, and almost all sea urchins reached full maturity by late July. In contrast, in groups reared at low temperatures (2.1–5.1℃), gametogenesis progressed slowly and over 60% of the sea urchins did not reach maturity even by early September. The feeding experiment also revealed that feeding with live *Saccharina diabolica* could increase the gonadal volume efficiently under low-temperature. These results demonstrate that low-temperature rearing, supplemented with feeding, is effective in suppressing gametogenesis to allow for the harvesting of high-quality sea urchins during the summer tourist season.

Böttger S. A., Devin M. G., and Walker C. W., 2006: Suspension of annual gametogenesis in green sea urchins experiencing invariant photoperiod – Applications for land-based aquaculture. *Aquaculture*, **261**, 1422–1431.

Sea urchin fisheries are valuable commercial resources in the United States with processed gonads sold in Japanese and American markets and maximum U.S. sales of $150M US dollars in 1996. Wild populations of sea urchins on all coasts of the U.S. have been heavily fished. Aquaculture of sea urchins in land-based facilities can help restore commercial populations and preserve this ecologically important herbivore. In this study, we used invariant summer photoperiod to prevent gametogenesis in the North American green sea urchins (*Strongylocentrotus droebachiensis*) maintained in a land-based aquaculture system and provided a commercially available formulated feed that promotes maximum growth of intra-gonadal somatic nutrient storage cells called nutrient phagocytes. Results were compared with individuals fed the same formulated feed under ambient photoperiod in cages in the ocean. Monthly samples of the gonads from both treatments were evaluated for gonad index, volume fractions of cellular constituents of the germinal epithelium, oocyte diameter and taste. Over the 5 months of this study, gonad indices increased significantly ($p < 0.001$) in both treatments from 4.8% ± 0.9 (all values ± SE) initially to 20.5% ± 2.1 under invariant and 23.2% ± 1.4 under ambient photoperiod with no significant difference between treatments ($p = 0.55$). Volume fractions of nutritive phagocytes increased to 80.3% ± 5.9 (initial 37.9% ± 7.1) in males and 71.0% ± 6.7 (initial 10.3% ± 4.0) in females ($p < 0.001$) only under invariant photoperiod. Nutritive phagocyte lengths increased under both photoperiod treatments, but the volume fraction containing nutrients was higher under invariant photoperiod. Volume fractions of gonial/gametogenic cells increased significantly ($p < 0.001$) only under ambient photoperiod from 20.4% ± 5.5 to 37.8% ± 1.8 in males and 0% to 22.6% ± 3.6 in females. The volume of fraction of residual oocytes from last year's oogenesis increased under invariant photoperiod while that of both residual and new oocytes increased under ambient photoperiod.

Residual oocyte diameters increase from 56.2 μm ± 2.2 initially to 93.5 μm ± 3.7 under invariant and those of residual and new oocytes to 126.0 μm ± 7.3 under ambient photoperiod. Invariant photoperiod yields gonads in both sexes of *S. droebachiensis* that do not initiate fall gametogenesis but attain large size as their nutritive phagocytes grow substantially in size. A Canadian study of wild-collected *S. droebachiensis* indicated that gonads taste best when they contain pre-dominantly nutritive phagocytes and not copious gametes, however gonad taste in our study was unsatisfactory suggesting that the only commercially available sea urchin diet requires modification to support commercial development of land-based aquaculture.

Nabata S., Hoshikawa H., Sakai Y., Funaoka T., Oohori T., and Imamura T., 1999: Food value of several algae for growth of the sea urchin, *Strongylocentrotus nudus* (in Japanese with English abstract). *Sci. Rep. Hokkaido Fish. Exp. Stn.*, **54**, 33-40.

Rearing experiments of the sea urchin, *Strongylocentrotus nudus*, were carried out during June to July, 1995 and June to August, 1996, using as food marine algae which settled and grew on the coralline flats after the removal of sea urchins. Feeding rate and growth rate at 17℃ of the sea urchin fed on *Laminaria, Undaria*, and *Costaria* were high and those for *Sargassum, Polysiphonia, Dictyopteris*, and *Desmarestia* were low in the single food item experiment. Among the algae supplied as food, the daily amount of food eaten was high in large-sized groups of the sea urchins, while the small-sized groups show the highest feeding rate. To examine any effect on gonad growth, we fed 3 algae, *Laminaria, Sargassum* and *Polysiphonia* to sea urchins. Two month later, the gonad index was found to be the highest in the *Laminaria* fed group. Among the algae fed, based on the gonad growth, the highest feeding rate and the highest growth rate, we estimated the *Laminariales* are the most nutritionally valuable algae for growth of *Strongylocentrotus nudus*.

水研センター研報，第40号，167－172，平成27年
Bull. Fish. Res. Agen. No. 40, 167－172, 2015

Long-term Outcomes in the Tech Transfer of Scallop Spat Collection Techniques, from Aomori Prefecture, Japan to Maine, USA

Dana L. MORSE*

Abstract: Improved management, aquaculture and stock enhancement of the sea scallop (*Placopecten magellanicus*) in Maine are examples of long-term benefits from a technology transfer project. In 1999, a delegation from Maine traveled to their sister-state of Aomori Prefecture in Japan to gain a firsthand view of the world-famous scallop industry of Mutsu Bay. While the trip was informative from many perspectives, the detail with most immediate application was the use of so-called 'spat bags' to capture competent larvae for stock enhancement and intensive aquaculture, and the way in which rotational management could be used to maximize production.

In subsequent years, over 100 fishermen in Maine used spat bags to capture juveniles, and the current progress made in aquaculture of sea scallops is largely due to the excellent success in collecting spat as a reliable seed supply. Maine's fishery is currently managed in rotation and area closures - sometimes at the request of the fishermen. Fishermen are also involved in discussions of spat collection for the purposes of stock enhancement. In addition, spat collectors have provided an invaluable window for fishermen to observe details of this early life stage, observations that they would not otherwise make. Overall, spat bags have been useful both directly and indirectly for well over a decade.

Many of these outcomes were not foreseen during the visit of 1999. Technology transfer often aims for immediate impact, but it seems likely that the normal case is that impacts and benefits may take many years to be observed. It is important to keep this in mind both in reporting to funders of various tech transfer efforts, but to appropriately gauge the long-term value of contemporary efforts.

Keywords: aquaculture, enhancement, *Placopecten magellanicus*, technology transfer

Maine's fishery for the sea scallop (*Placopecten magellanicus*) has traditionally been an important option for the state's fishermen; it was an open-access fishery until recently, and provided much-needed wintertime income. Direct on-the-water employment in the 2012/2013 fishing year amounted to 547 mobile-gear licensees (draggers), and 84 divers (Trish DeGraaf, ME Dept. of Marine Resources, pers. comm.). Landings have varied widely, from a historic high of 3.8 million pounds in 1981, to a low of 33,000 pounds in 2005 (Fig. 1). Only the adductor muscle, or meat, is landed.

Prior to 2007, regulation of the fishery was fairly limited, with no limit on the number of licenses, and a 3.5" (88.9mm) minimum shell height. Prompted by low landings and stimulated by examples of better management - such as discussed below - the Maine Dept. of Marine Resources engaged fishermen and scientists in a process to improve the landings and value of the fishery. Since then, several measures have been adopted, such as a larger minimum shell height of 4" (101.6mm), limited fishing days, a daily landing limit, rotational management, and closed areas, and to date, landings have improved (Fig. 1).

The process of industry engagement and inclusion into the management process was not always

2015年1月30日受理 (Received on January 30, 2015)
* Maine Sea Grant College Program and UMaine Cooperative Extension, 193 Clark's Cove, Darling Marine Center, Walpole, ME 04573 USA
E-mail: dana.morse@maine.edu

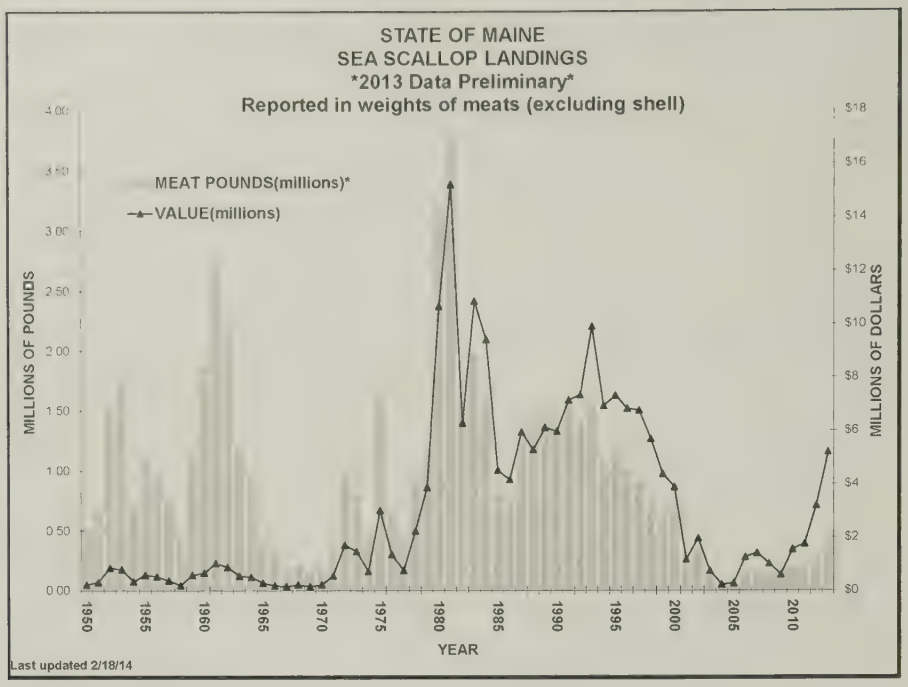


Fig. 1. Maine annual landings of scallop adductor muscles (meat).

smooth: the issues were highly contentious, and the bringing-together of industry with science and management meant that there was a good deal of learning to be done, toward and from one another. Conversely, it appears that these interactions have begun to pay dividends for all sides: management is more aware and responsive to industry perspectives, industry is more fluent in the science of scallop management and ecology, and science is being conducted on issues important both to fishermen and regulators.

Before the period reported in this document - 1999 and onward - engagement by fishermen and scientists in enhancement and aquaculture production was extremely limited and variable, with little in the way of planning or broader-scale thinking. Fishermen traditionally have experimented with enhancement through such actions as moving seed - meaning sublegal scallops, usually larger than 2" (5cm) - to a location they felt was protected or productive, or removing predators like crabs and starfish when they came up in the drag. Enhancement with seed smaller than 2" was very limited, and none performed in a structured fashion.

Scallop aquaculture has historically been opposed by many fishermen for two principle reasons: occupation of space that would normally be fished at one point of the year or other; and competition in the marketplace. A few small trials have yielded generally positive results though with several important caveats: work by Pottle and Hastings (2001) and Kuenstner (1996) - Maine polyculture study demonstrated that scallops would grow relatively quickly. The Pottle study also showed that whole scallops were a marketable item, but Kuenstner and Pottle both demonstrated the hurdles posed by algal toxins (saxitoxin, okadaic acid and domoic acid, principally) in selling products other than just adductor muscles.

Attitudes toward aquaculture have changed somewhat for many fishermen, however, with a major reason being the limited opportunities offered on the water in recent decades: limited access to licenses or resources have generally put fishermen 'in a box' where moving through several fisheries in the course of the year has been supplanted by access to only one or two fisheries, and therefore highly dependent upon them. The second reason concerns the subject of this paper: technology and information transfer becomes integrated in the knowledge, perspectives and ideas of the people who participate, and can support an adaptive stance toward current and future challenges.

Materials and Methods

Maine enjoys a sister-state relationship with the Prefecture of Aomori, Japan; the northernmost region of the island of Honshu. Delegations have passed with some regularity between Maine and Aomori, addressing such interests as energy, affordable housing, and fisheries. One such delegation traveled to Aomori in May of 1999, with the focus of learning about the highly-productive scallop industry there, one which has its' basis in aquaculture techniques, and one that has become a worldwide model of financial and social success. It was hoped that the group could identify aspects of the Japanese industry that would transfer well to the scallop fishery in Maine.

Between the 14th and 21st of May, ten representatives from Maine toured scientific facilities, processing plants, waterfront infrastructure, seafood markets, a fishermen's cooperative, and sea farms. Not unexpectedly, the delegation was impressed at the diversity, volume and vigor of the farming industry there. A brief list of conditions, opportunities and approaches that could potentially be beneficial to Maine ran as follows:

- A diverse suite of products from scallops would be beneficial for Maine, rather than just deriving income from the adductor muscle.
- Spat collection equipment and practices could be useful to stock enhancement and aquaculture in Maine. Techniques and equipment are directly transferrable.
- Collaboration with and support by scientists is beneficial to all involved; good monitoring for biotoxins is critical
- There is no difference between fisherman and sea-farmer in Japan: sea farmers are fishermen.

A full report was delivered to the Maine Aquaculture Innovation Center, detailing all aspects of the trip (Beal *et al.*, 1999).

In the years following the visit, several activities and projects were conducted in Maine, in an effort to build upon the knowledge gained.

Spat Collection: From 1999 to 2002 several meetings were held around the state, to familiarize fishermen with spat collection equipment and methodology. The emphasis was on searching for good spat collection areas, transferring captured seed to the wild, and educational connections with citizens and environmental groups (Fig. 2). As expected, fishermen quickly proved their expertise, and discovered that best spat collections were not done in the immediate coastal waters, but further offshore, near the limits of state jurisdiction (Fig 3). In these areas, counts were easily above 1,000 seed per collector, and frequently as high as 3,000. Many areas were found to result in collectors catching nearly exclusively scallops and with low fouling rates, whereas others yielded low scallop catch, high bycatch of other bivalves, and heavy fouling. It is estimated that between 1999 and 2002, over 100 fishermen had participated in some level of spat collection.

Fig. 2. Citizen environmental group aboard the F/V Lindsay Marie (Stonington, ME) to assist and learn about scallop spat collection and stock enhancement.

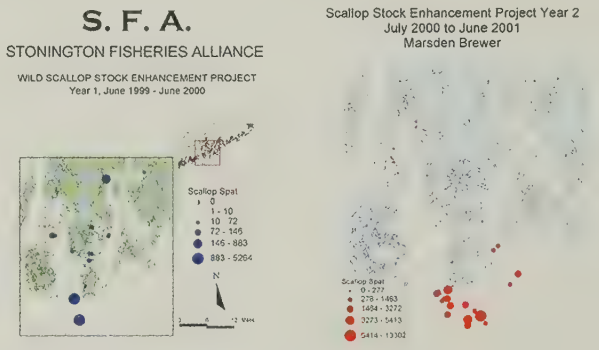


Fig. 3. Spat collections in 1999–2000 (left) and 2000–2001 in coastal Maine. Note that in the second year, most attempts were made further offshore because of prior success there: fishermen adapted rapidly to new knowledge.

Perhaps more importantly, fishermen found that spat collectors are most successful when they are observed regularly: the coast of Maine is heavily fished for lobster, and entanglements with lobster trap lines were common, unless the collector lines were tended. This was an important observation for the co-existence of spat collection with the lobster and other coastal fisheries. It was also during these years that the inclusion of spat collection, stock enhancement and aquaculture became better known to the state regulatory agency - the Maine Dept. of Marine Resources (DMR); this familiarity has proven to be very beneficial in the long run.

Stock Enhancement: Stock enhancement efforts in Maine can be generally characterized as haphazard, with a few exceptions. Efforts by the ME DMR and the Northwest Atlantic Marine Alliance (NAMA) were well coordinated, but of limited scope. Primary lessons from those efforts - which were both done with excellent collaboration between science and industry - were to reinforce the highly mobile nature of juvenile scallops, and the supposed high predation rates on seed less than 20mm or so (Schick and Feindel, 2005; Deese-Riordan, 2005). Unfortunately, these were general observations, and not thoroughly described in the detail desirable, due to limited ability to dive on the reseeded sites with the frequency necessary for such detail.

Other smaller efforts in stock enhancement were undertaken between 1999 and 2004; often amounting to a fishermen taking the spat from his collectors and deploying it in a favorite spot. The benefits of these activities have remained anecdotal and uncertain. The uncertainty over the success of stock enhancement led to the gradual decline of fishermen participating in spat collection. From roughly 2004 and onward, only a handful of fishermen per year participated; this area of study remains to this day one where industry interest would align well with scientific interest.

However, one area that has been of obvious benefit is the way in which spat collectors have allowed fishermen to observe and understand the earlier life phases of scallops; a subject that has been lightly discussed or considered previously. Spat collection led to conversations amongst fishermen and with scientists or managers about oceanography, predation, conservation, water quality and other topics, all of which entered the ensuing discussions about management of the fishery and the scallop resource. Similarly, spat collectors also spurred fishermen's interest in aquaculture: seeing 2000 small scallops in a spat collector immediately led them to consider what it would be like to raise them in cages. Both of these phenomena have played a large role over time in the evolution of scallop management in Maine, and the beginnings of an aquaculture sector.

Aquaculture: The investigation of sea scallops as a candidate for aquaculture in Maine has been a story of stops and starts. From 1999 to 2000, a trial of cage culture for scallops was undertaken by Tom Pottle, who was one of the delegates from the 1999 trip, and a fisherman from the Cobscook Bay region. His project successfully demonstrated reasonable growth rates, useful equipment and husbandry practices, and positive feedback from test sales into the marketplace (Pottle, 2001). It also underscored the importance of thorough testing for toxic algae, and presence of algal toxins in scallop tissues. Mr. Pottle did not continue his project past the funded time frame, though has remained optimistic about the chances for scallop aquaculture in Maine.

From 2000 through approximately 2010, scallop aquaculture was at a virtual standstill in Maine; the risks associated with sales of live or roe-on product were prohibitive for state regulators. In recent years however, conversations between industry and the regulatory community have increased, with both sides joining in an effort to explore the possibility for sales of roe-on or live/whole product. A current project involves five pilot scallop farms, with excellent growth observed thus far (up to 0.19mm/day), and low mortality (Fig. 4). Conversations continue regarding the protocols necessary to guarantee product and public safety - but they are occurring and all parties have felt optimistic for future development.

To date, first sales of live scallops are anticipated in 2014, accompanied by sales of seed from spat collectors to new growers. Project details located at: http://www.seagrant.umaine.edu/research/projects/dv/scallop-trials

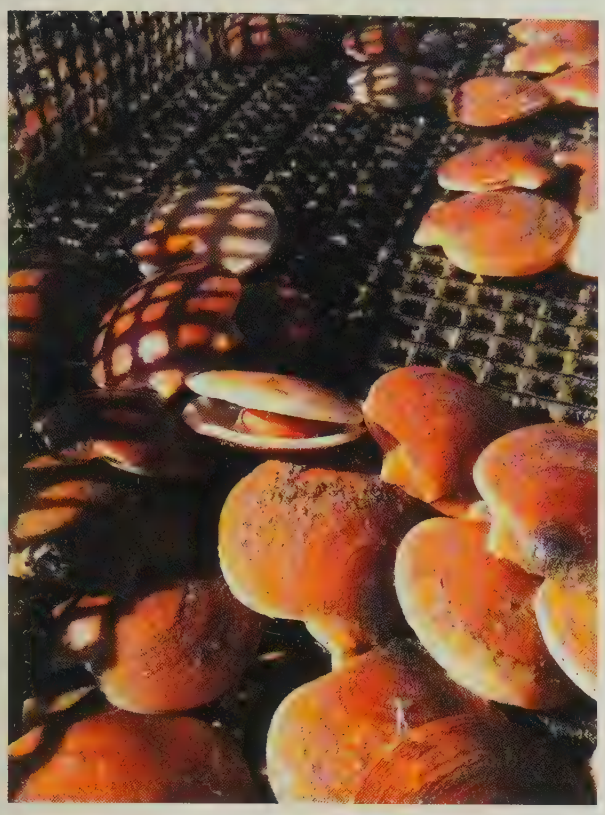


Fig. 4. Scallops growing in a cage during the pilot project; Blue Hill, ME

Results and Discussion

Technology transfer clearly yields both short-term and long-term results. While it may be possible to anticipate or predict the near-term benefits and activities that stem from a technology transfer activity, real predictions over the longer term are much less achievable, other than to say that they will exist. Documenting such developments is a worthwhile endeavor however, because of the local relevance but also because of the larger implications for future efforts: funding for travel is usually difficult, and when good documentation is available on the value of such travel, it improves the rationale for proposals involving travel to support technology transfer. Therefore, while it's very difficult to anticipate long-range outcomes, it is of value to note them as they do become apparent.

In the case of the 1999 trip to Aomori, three principal areas of impact can be easily identified. These topics - which are closely linked - are: biology and ecology, management, and production.

In the category of Biology and Ecology, several items come to mind. The Aomori visit supported an improved understanding of biology and ecology of scallops, compared to the level of understanding prior, particularly by industry. By focusing on the deployment of spat collectors and observing the catches, fishermen started to better understand important factors such as proximity of spawners to one another, the influences of oceanographic conditions (currents, tides, temperatures, etc) on larval dispersal and settlement, and in particular, the challenges faced by very young post-set scallops. In fact, the simple identification of young scallops improved a good deal: fishermen have often mistaken so-called 'jingle shells' (*Anomia simplex*) as young scallops; spat collectors have allowed fishermen access to the very smallest sizes, such that identification is now much more accurate - and questions have naturally followed those observations.

The concepts of rotational management have been reinforced; many had focused on the management of offshore scallops on George's Bank as the primary example, but the Japanese example has figured into management discussion. Today, Maine's inshore scallop fishery is managed in rotation, in some measure due to a changed way of thinking by fishermen, scientists and managers resulting from the Aomori visit (Fig. 5). Other issues such as mandatory stock enhancement and creation of spawner sanctuaries have been included in the management discussion and although not included in regulations, have been a noteworthy step about how fishermen and managers have discussed the responsibilities and possibilities in supporting a healthy resource and fishery.

Production remains an area where the Aomori visit has had a profound influence, all starting with the technology of spat collection. Stock enhancement continues to gather support from fishermen when the subject arises, and it's likely that more work will be done in this area, and with greater scientific scrutiny than before, which would permit a better evaluation of effectiveness. An aquaculture industry has begun to develop in the state as well with small sales of the first crop (and scallop seed from collectors) expected later in 2014. Moreover, the tenuousness of the lobster, shrimp and groundfish industries have made commercial fishermen more

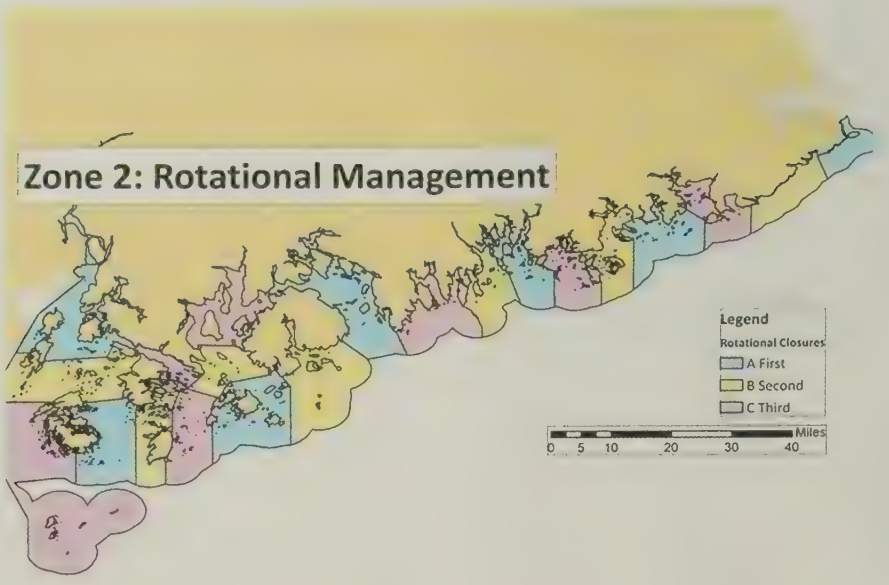


Fig. 5. Ten-year rotational management plan for the Maine scallop fishery; in this case the eastern zone.

open to the possibility of aquaculture as a way to generate income, and thus the integration of the fishing and aquaculture industries has a pathway for progress, through the culture of sea scallops.

The quantification of impacts is more difficult, though not impossible. Landings for scallops have increased in recent years, and surveys of the areas closed as part of the rotational management approach have provided strong indications that management plays a large role in the improvements. Therefore, landings and licenses are two metrics that can be easily identified. As the aquaculture industry continues to advance, it is straightforward to record economic activity and the number of farmers as measures of success. These data are important in the sense of tracking the influence of the original information.

The natural complement to the numbers themselves (sales, licenses, leases, etc) is the use of anecdotes to tell the story of what happened over time. When taken together, the quantifiable data and the qualitative assessments, the stories can create an understandable and informative landscape of the value of technology transfer in both the short and long terms. In fact, both are necessary to fully understand the impacts. In the case of the Aomori trip of 1999, the payoffs became apparent rapidly, and it appears that benefits will continue well into the future.

References

Brian F. B., (ed. and principal writer). 1999: The culture of Japanese Scallops (*Patinopecten yessoensis*), Report of the Maine Delegation to Aomori Prefecture, Japan, May 14-21, 1999. The Maine Aquaculture Innovation Center, Orono, Maine, USA.

Deese-Riordan H., 2005: Saco Bay Scallop Stock Enhancement Project. Final Report to the Northeast Consortium. Project Development Grant #03-194. 31p.

Kuenstner S., 1996: Polyculture of sea scallops suspended from salmon pens. New England Fisheries Development Association, final report to the NOAA Saltonstall-Kennedy Program. Project No. 93-NER-009, Grant No. NA46FD0327. 91p.

Pottle T., and Hastings M., 2001: Sea Scallop Demonstration Project. Final Programmatic Report, National Fish and Wildlife Foundation. 10p

Schick D. F., and Feindel S. C., 2005: Maine Scallop Fishery: Monitoring and Enhancement. Final Report to the Northeast Consortium, September, 1, 2005. Award No. 013-138-2051-30. 73p.

水研センター研報，第40号，173－177，平成27年
Bull. Fish. Res. Agen. No. 40, 173－177, 2015

Oyster Culture in Hokkaido, Japan

Natsuki HASEGAWA[*1], Toshihiro ONITSUKA[*2], Satoru TAKEYAMA[*3], and Kimihiko MAEKAWA[*4]

Abstract: The Pacific oyster, *Crassostrea gigas* is the one of the most commercially important aquaculture species in the Japanese fisheries industry and is cultured in various Japanese coastal areas including Hokkaido. The Saroma Lake and the Akkeshi Bay with estuary, which face Ohotsuku Sea and Pacific Ocean, respectively, are major oyster production areas in Hokkaido with total annual production of about 700 tons a year. The seedling spat supplied from Miyagi known as Miyagi seedlings are widely used in Japanese oyster culturing. Therefore, when the catastrophic tsunami on March 11, 2011 damaged the Miyagi fisheries, many oyster culturing areas were heavily affected. Moreover, introduction of seedlings from geographically separated population have risks of invasion of diseases and alien organisms as hitchhiking species. Using seedlings that originated from local populations in each area is one of the approaches for decreasing some risks. For example, in the Akkeshi area, the artificial seedling spats collected from the locally protected adults are also used for aquaculture, which are marketed as the value-added oysters with shell "Kaki-Emon" and popular among consumers as local special products.

Key words: Pacific oyster (*Crassostrea gigas*), Miyagi seedlings, Saroma Lake, Akkeshi Bay

The Pacific oyster, *Crassostrea gigas* is the one of the most commercially important aquaculture species in the Japanese fisheries industry with harvest of around 200, 000 metric tons a year, the same as the Japanese scallop *Mizuhopecten yessoensis* (Ministry of Agriculture, Forestry and Fisheries, Minister's Secretariat, 2012). Although a lot of scallops were exported to other countries including the United States, most oysters are consumed domestically. Oyster culturing in Japan is reviewed by Inui (2013). The Oyster is cultured using raft or long line hanging method. Hiroshima and Miyagi prefectures are the main production areas in Japan, and there are also many other production areas along Japanese coastal area. Most of these areas use oyster seedlings which are naturally collected in Miyagi using scallop shell collectors and are known as Miyagi seedlings. Therefore, when the catastrophic tsunami on 2011 damaged the Miyagi fisheries, many oyster farmers were heavily affected because of uncertainty of seedling supply (Tanabe, 2012)

Hokkaido, a northern island of Japan is one of the premier oyster culturing areas with production of 700 metric tons a year (Marinenet Hokkaido; http://www.fishexp.hro.or.jp/marineinfo/internetdb/index.htm), using primarily Miyagi seedlings. Hokkaido has two main production areas, Saroma Lake and Akkeshi Bay and estuary (Fig. 1). Saroma Lake and Akkeshi Bay face towards the Ohotsuku Sea and the Pacific Ocean, respectively. These areas have similar production scales and Oyster industry history. In the early days in Hokkaido natural oyster beds were very productive in these areas and the oysters were harvested from there (Inukai and Nishio, 1937). However, natural oyster resources were lost around

2015年1月30日受理（Received on January 30, 2015）
*1 National Research Institute of Aquaculture, Fisheries Research Agency, 422-1, Nakatsuhamaura, Minamiise, Watarai, Mie, 516-0193, Japan
E-mail: hasena@fra.affrc.go.jp
*2 Hokkaido National Fisheries Research Institute, Fisheries Research Agency (Kushiro Laboratory), 116 Katsurakoi, Kushiro, Hokkaido, 085-0802, Japan
*3 Akkeshi Oyster Hatchery, 1-1, Wakatake, Akkeshi, Hokkaido, 088-1118, Japan
*4 Aquaculture Cooperative of Saroma Lake, Sakaeura, Tokoro, Hokkaido, 093-0156, Japan

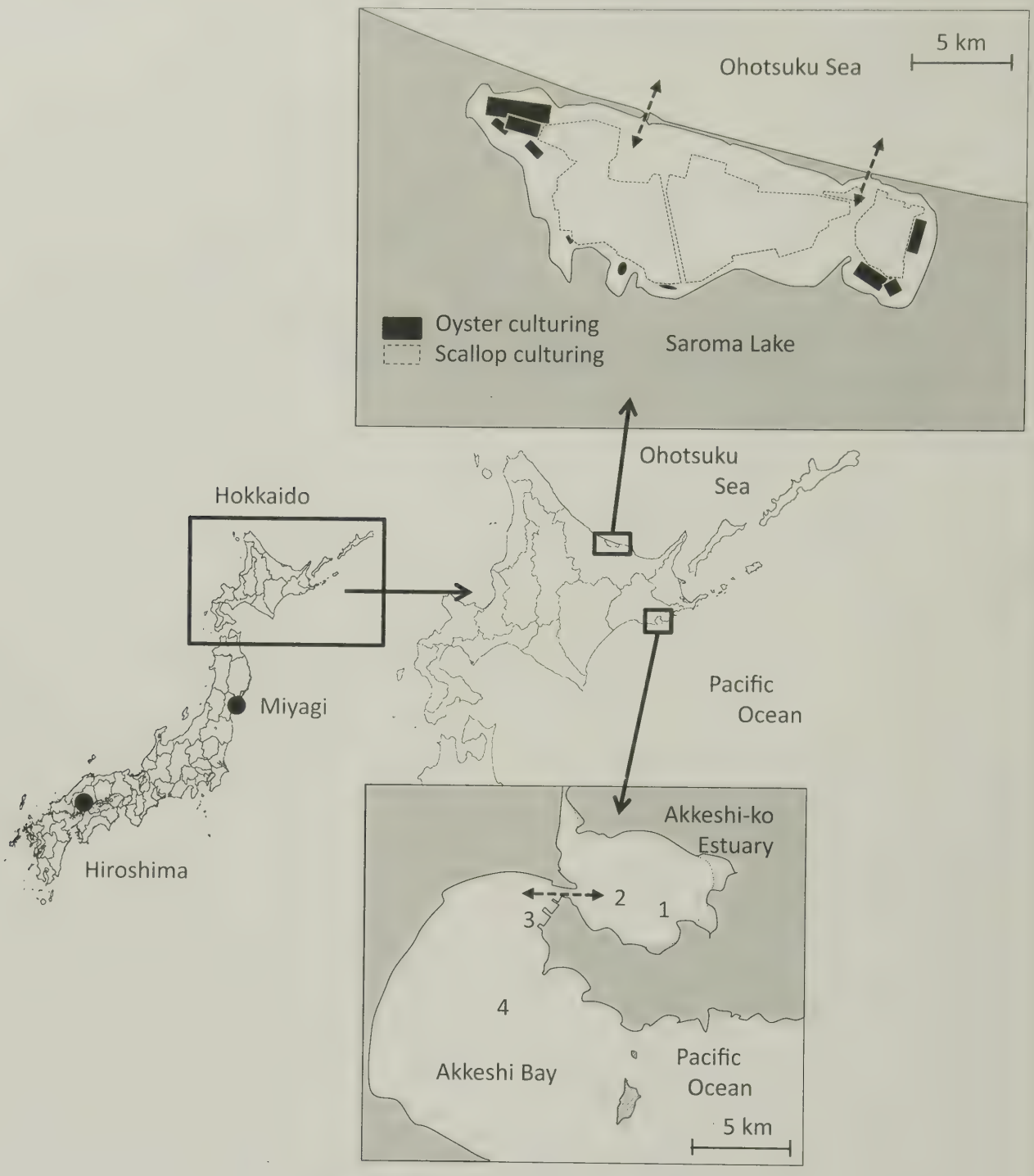


Fig. 1. Main Oyster culturing areas in Hokkaido, Japan.

the 1930s and since then the oyster industry has relied on Miyagi seedlings.

Features of oyster and scallop culturing in Saroma Lake

Ground and suspended culture of scallops are the main fisheries in the Abashiri Sub prefecture with Saroma Lake in Hokkaido where production reaches 150,000 metric tons a year. Most areas of Saroma Lake are utilized for scallop culture and unsuitable areas for scallop culture due to shallow depth and high water temperatures during summer are used for oyster culture (Fig. 1). In the lake, scallop culture is a major fishery (> 77% of total catch), however, most culture activities must be stopped

during winter due to lake freezing. Oyster fisheries contribute a little (< 1%) to increase the diversity of fisheries and reduce dependency on scallop culture, but oyster culturing brings jobs to farmers in winter such as shucking because oyster's in this area are mainly composed of age 1+ and so small oysters are shipped as a shucked product. Moreover, older fishermen have special feeling for oyster culturing because oyster fishing had been prospered in the lake until 1928 when the continuous channel was opened between Lake and cold Ohotsuku Sea and the oyster could not reproduce in stable (Nishihama, 1994).

Features of oyster culturing in Akkeshi Bay and Estuary

The most important feature of Akkeshi is the topography (Fig. 1), Akkeshi has two different ecosystem types; Akkeshi Bay and the Akkeshi-ko estuary. Akkeshi Bay faces the Pacific Ocean where a cold subarctic ocean "Oyashio" current flows, while the Estuary is shallow (average depth: 1.5 m). The continuous measurement of surface water temperature shows that the temperature in this Estuary increases earlier than in the Bay from spring to summer, reaching temperatures higher than 25 ℃. However, water temperature in the Bay is around 20 ℃ in late summer (Fig. 2). Culture experiments using single-seedling oysters showed variations of growth rate and nutritional condition index between oysters from the Bay and the Estuary. The ratio of flesh wet weight to whole body weight of oyster, which is one of the indices of oyster nutritional condition was lower in the estuary than the bay throughout the experiments (Fig. 3a and b). The specific growth rate of shell height was higher in the Estuary than the Bay in early summer (Fig. 3c and d). The early increase in temperature from spring to summer enhances oyster growth and maturation and early spawning occurs in the Estuary. These features of both culture areas are recognized by farmers and efficiently utilized. The fishermen use the Estuary as the warmer area for enhancing oyster growth and maturation with early spawning. Otherwise, the Bay has a greater surface area, water volume, and food availability than the Estuary, which contributes to improved body conditions of oysters before shipping seasons.

Therefore, fishermen use these two areas properly, and Akkeshi oysters have some advantages to other culturing areas. But carrying capacity is limited by the topography of the shallow and small estuary. To avoid this problem, single-seed oysters are cultured in Akkeshi, which are suitable for farming in shallow areas. In the hatchery, larvae are collected from local adult spawns and settled on small oyster shell pieces individually as single-seedling oysters. The juvenile

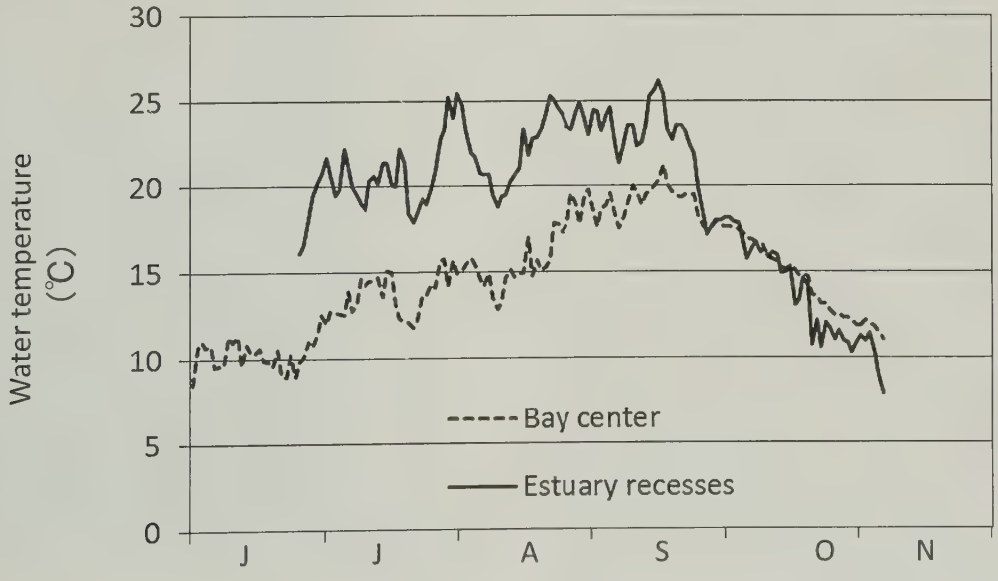


Fig. 2. Fluctuation of water temperature at Akkeshi Bay and Estuary, Japan during 2012.

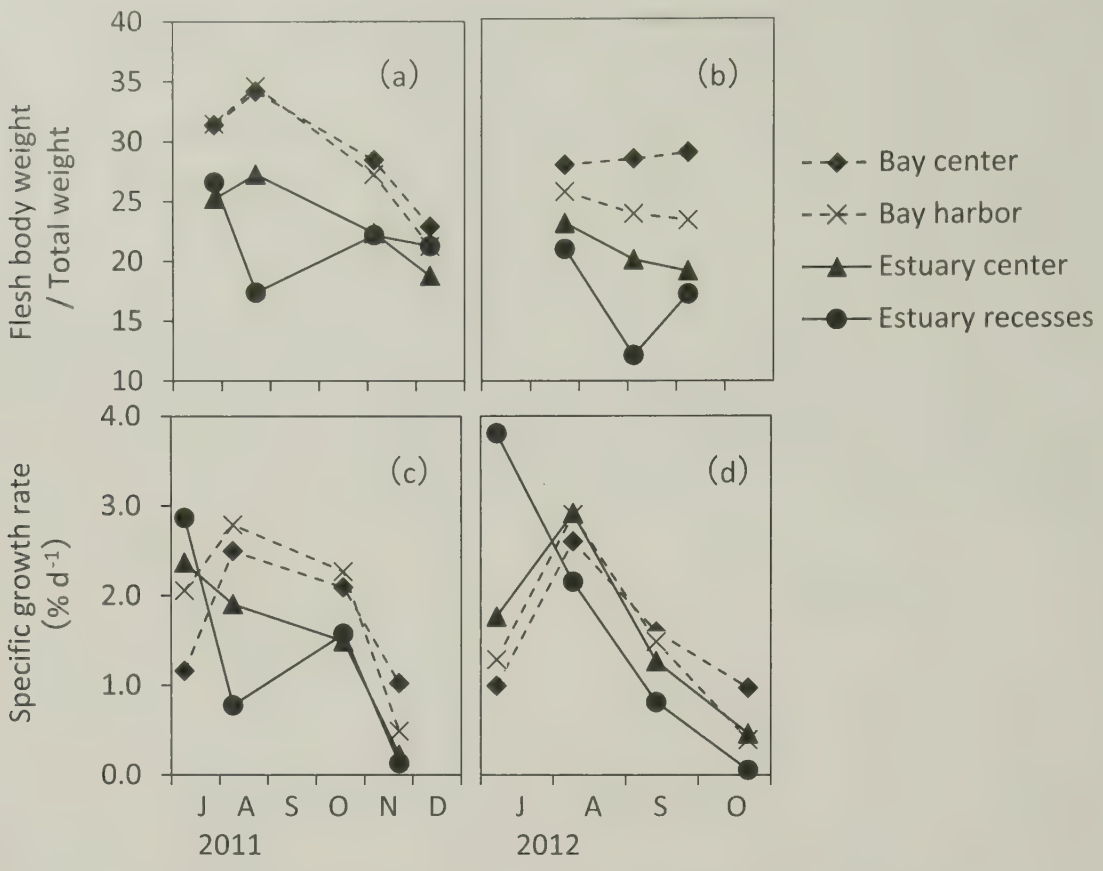


Fig. 3. Temporal and spatial variation in ratio of fresh wet weight to total weight to whole weight (a and b) and specific growth rate of shell height (c and d) of *Crassostrea gigas* in Akkeshi Bay and Estuary, Japan.

oysters are cultured in the bay and the estuary using baskets. By using basket rearing, more oysters can be cultured in shallow areas such as Akkeshi-ko estuary. And basket hanging keeps oysters in the water column away from pollution on the estuary bottom. Moreover, through basket hanging, single-seed oysters have deeper shell breadth than oysters on scallop shells. These oysters are marketed as value-added oysters with shell "Kaki-Emon" and are popular among consumers as local special products (Fig. 4). Akkeshi town is aiming to be known as "Oyster town" using single-seed oysters to promote tourism and sightseeing, and to develop brand strength of other fisheries products. However, the yield of single-seed oysters was lower than that of Miyagi-seedling oysters at the beginning of this culturing, because the standard culturing methods for Miyagi seedlings were not suitable for single-seed oysters. Therefore, the culture of single-seedling oysters does not increase as much as the research and promotional organizations expected, even today.

Fig. 4. PR poster of local premium oyster in Akkeshi "Kaki-Emon".

Problems facing oyster culturing in Hokkaido

Miyagi-seedling has been widely used in Japanese local production areas including Hokkaido because most of the seeding are naturally collected using scallop shells with low costs and a stable source provided by the specialized farmers in Miyagi prefecture. These Miyagi oysters have faster growth potential, and thus are preferred by many oyster farmers. Even though there is wide-scale use of Miyagi-seedlings throughout the area, genetic differences can be detected among some local/wild populations in Japanese local production areas (Usuki *et al.*, 2002). The tsunami in 2011 exposed the risks of excessive dependence on Miyagi seedling supplies. Moreover, introduction of seedlings from geographically separated populations have risks of introducing diseases and alien organisms as hitchhiking species. For example, invasive ascidians were also recorded in some bivalve culturing areas in Japan. In particular, *Ascidiella aspersa* strongly affect the scallop culturing in Funka Bay, Hokkaido (Nishikawa *et al.*, 2014). The paramyxean parasite, *Marteilioides chungmuensis*, has negative impacts on the oyster industry in some Japanese production areas with heavily infected oyster's showing abnormal tissue (Ito *et al.*, 2002). Moreover, the potential transport of harmful algae together with bivalves has also been reported (Matsuyama *et al.*, 2010). Using local seedlings is one of the approaches to decreasing these risks. After the Miyagi seedling crisis, the use of local populations is beginning in some production areas. However, similar to the experience in Akkeshi, it is necessary to improve culturing techniques and management for culture of local seedlings because they have different characteristics. Moreover, sales strategies of "local oyster" with premium price are also important to promoting use of local seedlings to compensate for additional cost and effort.

Acknowledgements

This research was partly supported by annual research grants "Study of coastal shellfish fisheries to support the recovery from tsunami damage in eastern Hokkaido" and grants-in-aid for Scientific Research "Analysis and future prediction of coastal ecosystem" provided by the Hokkaido National Fisheries Research Institute, Fisheries Research Agency of Japan.

References

Inui M., 2013: Enclopedia · Oyster culturing in Japan. Suisanshinkou, **544**, Tokyo Fisheries Promotion Foundation, Tokyo, 120pp. (In Japanese)

Inukai T., and Nishio S., 1937: A limnological study of Akkeshi Lake with special reference to the propagation of the oyster. *Journal of the Faculty of Agriculture, Hokkaido Imperial University*, **40**, 1-33. (In Japanese)

Itoh N., Oda T., Ogawa K., and Wakabayashi H., 2002: Identification and development of a paramyxean ovarian parasite in the Pacific oyster *Crassostrea gigas*. *Fish Pathology*, **37**, 23-28.

Matsuyama Y., Nishitani G., and Nagai S., 2010: Direct detection of harmful algae from the oyster spat and live fish transporting trailer Proceedings of 13th International Conference on Harmful Algae, 185-189.

Ministry of Agriculture, Forestry and Fisheries, Minister's Secretariat 2012: Annual report of catch ststistics on fisheries and aquaculture (In Japanese)

Nishihama Y., 1994: Scallop fisheries in Ohotsuku Sea. Hokkaido University Press, Sapporo 218p.

Nishikawa T., Oohara I., Saitoh K., Shigenobu Y., Hasegawa N., Kanamori M., Baba K., Turon X., and Bishop D. D. J., 2014: Molecular and morphological discrimination between an invasive ascidian, *Ascidiella aspersa*, and its congener *A. scabra* (Urochordata: Ascidiacea). *Zool. Sci.*, **31**, 180-185.

Tanabe T., 2012: The Natural Seed Collection of Pacific Oyster, *Crassostrea gigas*, After the Great East Japan Earthquake on 3.11 *Miyagi Pref. Rep. Fish. Sci.*, **12**, 47-59. (In Japanese)

Usuki H., 2002: Evaluation of characteristics and preservation of Pacific oyster, *Crassostrea gigas*, in view of the genetic resources. *Bulletin of Fisheries Research Agency*, **40**, 40-104 (In Japanese with English abstracts).

水産総合研究センター研究報告

Bulletin of Fisheries Research Agency (No. 40)

Pacific Bluefin and North Pacific Albacore Tuna Age Determination Workshop
Editing Board of Special Issue
Tamaki Shimose

The 41st Scientific Symposium of the UJNR Aquaculture Panel
"Advanced Aquaculture Technologies"
Editing Board of Special Issue
Koichi Okuzawa, Paul G. Olin,
Michael B. Rust and Emily M. Trentacoste

Published by Fisheries Research Agency
Queen's Tower B 15F, Minato-mirai 2-3-3
Nishiku, Yokohama, Kanagawa 220-6115, Japan

President of FRA: Masanori Miyahara

Printed by BIKOHSHA INC.

Copyright© (2015) by FRA

BULLETIN OF
FISFERIES RESEARCH AGENCY

No. 40
March 2015

Bull.Fish.Res.Agen.

CONTENTS

(Symposium)
Pacific Bluefin and North Pacific Albacore Tuna Age Determination Workshop 1

The 41st Scientific Symposium of the UJNR Aquaculture Panel "Advanced Aquaculture Technologies" 19